The Synthesis and Applications of Disulphide Containing Oligonucleotides

by

Adrian J. Wilkinson

A thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

1994

To

my

Parents

Acknowledgements

I would like to thank:

–Professor T. Brown, my supervisor, for his constant encouragement and enthusiasm.

–Dr. M. D. Edge, my industrial supervisor, for his advice and ideas.

–The Brown group, both past and present.

–The staff at the Zeneca Pharmaceuticals Alderley Park site, particularly Dr. D. Holland, for making me feel so welcome.

–OSWEL DNA Unit for providing equipment and reagents.

–The SERC and Zeneca Pharmaceuticals for funding my work.

–Edinburgh University for the use of its NMR spectra, mass spectra and CHN microanalyses facilities.

ABSTRACT

The poor cellular uptake properties of oligonucleotides represent a major drawback to the antisense approach to antiviral therapy. To circumvent this problem, bulky lipophilic molecules such as cholesterol which interact with cell membranes have been bonded to the 5'-terminus of potential antisense antiviral oligonucleotides. A novel disulphide containing phosphotriester monomer has been synthesised which possesses oligonucleotide coupling efficiencies which are superior to those obtained from existing disulphide monomers. This dipropyl disulphide linker, which should be stable outside the cell but vulnerable to disulphide cleavage by thiols contained in the cell, has been introduced between the lipophilic groups and oligonucleotides. The oligonucleotides should thus be released from the lipophilic groups once endocytosis has occurred.

Similarly, the dipropyl disulphide linker has been introduced between a biotin group and an oligonucleotide. Quantitative disulphide cleavage can be achieved by the addition of dithiothreitol. Thus oligonucleotides bearing cleavable biotin moieties have been synthesised, employing a far more facile and high yielding process than the current method of choice. This approach has been extended to the synthesis of a novel amino functionalised disulphide linker for the production of the first cleavable aminolink oligonucleotides.

Cleavage of the disulphide bond in the dipropyl disulphide linker produces a propyl thiol group at the 5'-end of an oligonucleotide. A diethyl disulphide linker has been synthesised which produces an ethyl thiol group on the oligonucleotide after disulphide cleavage. Under basic conditions, the oligonucleotide can eliminate mercaptoethanol to give a 5'-phosphate group. Thus a simple, high-yielding method for the synthesis of terminal thiol and terminal phosphate functionalised oligonucleotides has been developed, offering a viable alternative to existing routes to these functionalities.

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ABBREVIATIONS

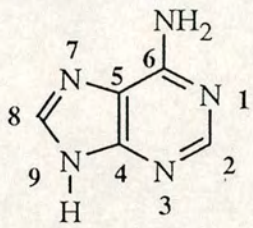
A	adenine
AIDS	acquired immune deficiency syndrome
AZT	3'-azido-2',3'-dideoxythymidine
C	cytosine
C2SS	diethyldisulphide group
C16	hexadecyl group
C35	1,2-di-O-hexadecylglyceryl group
C35-C6	aminohexyl linked 1,2-di-O-hexadecylglyceryl group
Chol	cholesteryl group
Chol-C6	aminohexyl linked cholesteryl group
CPG	controlled pore glass
dA	2'-deoxyadenosine
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
dC	2'-deoxycytidine
DCC	dicyclocarbodiimide
DEC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
dG	2'-deoxyguanosine
DIPEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	N,N-dimethylformamide
DMTr	4,4'-dimethoxytrityl
dT	2'-deoxythymidine
DTT	dithiothreitol
FAB	fast atom bombardment
FMOC	9-fluorenylmethoxycarbonyl
G	guanine

HEG	hexaethyleneglycol group
HIV	human immunodeficiency virus
HSV1	herpes simplex virus 1
IAF	5-iodoacetamidofluorescein
MMTr	4-monomethoxytrityl
mRNA	messenger RNA
MSNT	1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole
NAP	nucleic acids purification
NHS-SS-Biotin	sulphosuccinimidyl-2-(biotinamido)ethyl-1,3'-dithiopropionate
OD	optical density
PCR	polymerase chain reaction
PVCl	pivaloyl chloride
SPDP	N-succinimidyl-3-(2-pyridyldithio)propionate
SS	dipropyldisulphide group
T	thymine
TBDMS	*t*-butyldimethylsilyl
TBP	n-tributylphosphine
TCA	trichloroacetic acid
TEA	triethylamine
TEAB	triethylammonium bicarbonate
TETD	tetraethylthiuram disulphide
THF	tetrahydrofuran
THP	tetrahydropyranyl
tlc	thin layer chromatography
Tosyl	*p*-toluenesulphonyl
TPSCl	triisopropylbenzene sulponyl chloride
tRNA	transfer RNA
U	uracil

1 INTRODUCTION

1.1 General Introduction to DNA Chemistry

Much of the knowledge we possess today concerning the structure and function of DNA is taken for granted. However, most of the important breakthroughs in DNA chemistry have occurred relatively recently. In 1933, Thannhauser and Klein[1] first obtained the four deoxyribonucleotide components of DNA. The structures were confirmed by the first dinucleotide synthesis by Todd[2] in 1955, following the development of methods of phosphorylation.



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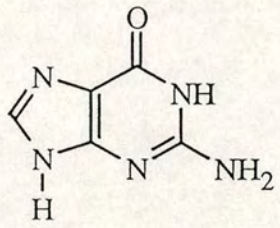

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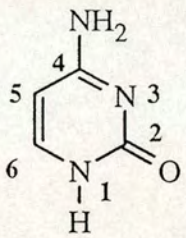

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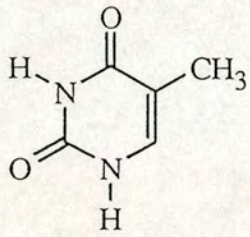

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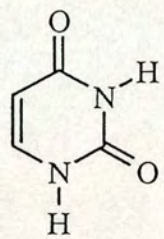

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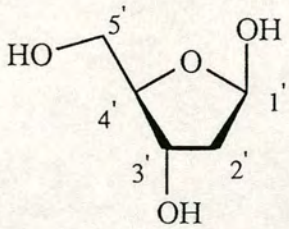
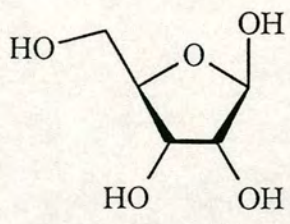


2'-deoxy-D-ribofuranose



D-ribofuranose

Figure 1.1

In 1953, Watson and Crick,[3] discovered the complementary double-helical structure of DNA and with it came understanding of the process of replication. Once the genetic code had been elucidated,[4-6] modern DNA chemistry began.

All DNA nucleotides are composed of a phosphate, a deoxyribose sugar and one of four main nitrogen heterocyclic bases (***Figure 1.1***): adenine (A), guanine (G), cytosine (C) and thymine (T). In RNA nucleotides, the pyrimidine, uracil (U), and the D-ribose sugar occur instead of thymine and 2'-deoxyribose respectively. The nucleosides are linked by 3' to 5' phosphodiester groups with the bases attached to the sugar at the 1' position in the β-configuration (***Figure 1.2***).

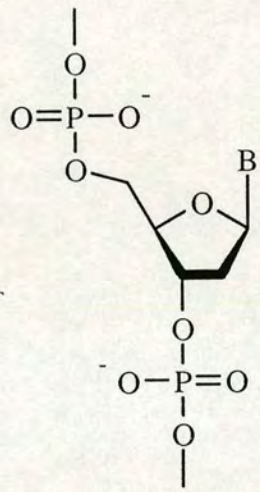


Figure 1.2

Where B = A, G, C or T

The high nitrogen and oxygen content of the heterocyclic bases provides them with considerable potential to form hydrogen bonds and ionisable phosphate groups engender them soluble in aqueous rather than organic solvents. The hydrogen bonds between thymidine and adenosine and guanosine and cytidine (***Figure 1.3***) stabilise the so called Watson-Crick base pairs.[3] The specific base pairs formed are mainly responsible for holding together the double helical structure of DNA as an antiparallel duplex. Although many conformations of DNA have been identified,[7-9] the most important are the right handed double helices of A-DNA and B-DNA. Duplex

stability can be measured in terms of the melting temperature, the temperature at which 50% of the duplex has denatured.

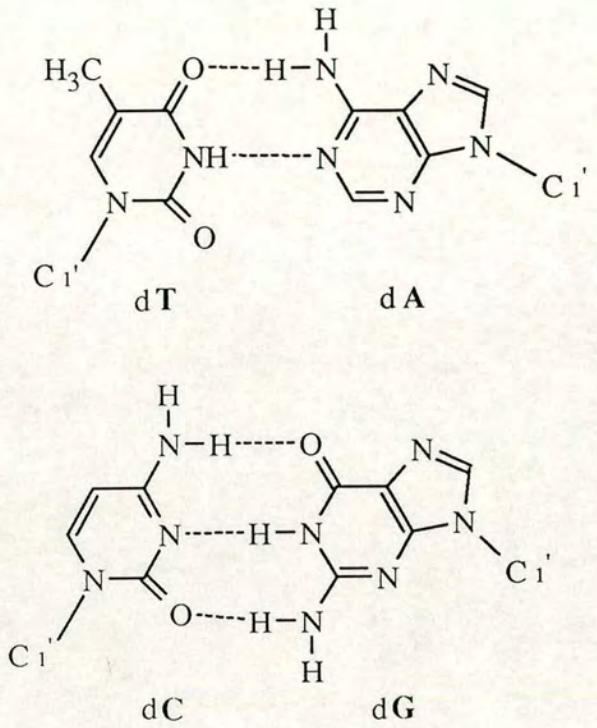


Figure 1.3 : Watson-Crick Base Pairs

The primary function of DNA is to store the genetic code for each particular organism. By the process of replication, DNA is copied so that each daughter cell has its full complement of DNA after cell division. The transcription of DNA leads to the formation of a complementary strand of messenger RNA (mRNA) which is transported to the ribozomes where protein synthesis occurs. Transfer RNA (tRNA), a short strand of RNA less than 100 nucleotides in length, carries individual amino acids to the ribozome where they form peptides and proteins of sequence determined by the mRNA. Every amino acid is coded for by a codon, a specific, 3 base, non-overlapping triplet, on the mRNA. Thus RNA plays an essential role in the functioning of the cell.

1.2 Chemical Synthesis of Oligonucleotides

The demand for a rapid, high yielding process for the synthesis of short strands of DNA, known as oligodeoxyribonucleotides or, more generally, oligonucleotides, is enormous. They are used as primers in DNA sequencing or amplification, mutagenic agents to alter the sequence of genes, probes or labels in DNA detection, isolation and diagnostics and as potential therapeutics. Any chemical process for oligonucleotide synthesis has to be compatible with the numerous functional groups present and the sensitivity of certain parts of the molecule (e.g. the acid lability of the glycosidic sugar to base bonds). The basic strategy in oligonucleotide synthesis is to form a phosphodiester bond between the 3'-hydroxyl group of one nucleoside and the 5'-hydroxyl group of the other.

1.2.1 Solid Phase Synthesis

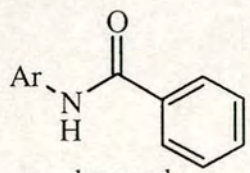
The breakthrough of Merrifield[10] in the synthesis of peptides on a solid support led to a similar technology being applied to oligonucleotide synthesis.[11] A nucleoside is bound, usually through the 3'-hydroxyl group, via a linker to an insoluble solid support to allow 3' to 5' chain extension. A sequential addition of monomers is carried out until the desired oligonucleotide is obtained which is then cleaved from the solid support. Controlled pore glass (CPG) beads,[12] rigid and non-swellable, have proved the most useful solid support. These are usually derivatised with aminopropyl groups to extend the active sites away from the solid surface and ensure accessibility of the growing DNA chain to all reagents. The CPG is usually linked to the oligonucleotide by a succinyl ester group. The succinyl linkage is cleaved by base hydrolysis using ammonium hydroxide.

In solid phase methodology, all reagents employed in chain assembly are removed simply by washing the CPG and filtering. As a result, all reagents can be used in excess and yields are high. There is no need for purification steps during chain

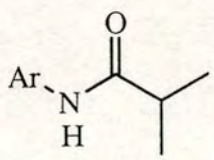
assembly and this allows more rapid syntheses. In the 1980s, solid phase synthesis was automated and the phosphite triester approach is now the routine method of oligonucleotide synthesis. The primary drawback of solid phase synthesis is the limitations of scale; the CPG resin can only contain a limited number of active sites, between 10 and 60μmolg^{-1} loading, above which the individual growing chains are susceptible to steric crowding. This is currently an area of active research.

1.2.2 Protecting Groups

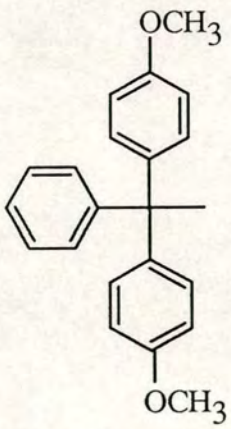
The exocyclic amino groups of the A, G and C bases must be protected during oligonucleotide synthesis to prevent undesirable side-reactions. The protecting groups have to be stable throughout oligonucleotide chain assembly, but must be cleavable once the synthesis is complete i.e. they are semi-permanent protecting groups. Khorana[13,14] introduced acyl protecting groups in the 1960s and indeed this type of base protection is still the most popular. The benzoyl group is used to protect both adenine and cytosine and the isobutyryl group to protect guanine (***Figure 1.4***). Thymine requires no protection as it does not contain an exocyclic amino group. The protecting groups are removed by heating in ammonium hydroxide for 5 hours at 55^{o}C.



benzoyl



isobutyryl



4,4'-dimethoxytrityl (DMTr)

Figure 1.4

The protecting group of choice for the 5'-hydroxyl position of the deoxyribose sugar, a temporary protecting group, is the 4,4'-dimethoxytrityl (DMTr) group (***Figure 1.4***), again introduced by Khorana.[15] It is sufficiently acid labile that an acid such as trichloroacetic can rapidly remove it. The 4,4'-dimethoxytrityl cation thus released has a bright orange colour (495nm) which can be used to determine the success of the coupling reaction.

1.2.3 Methods of Oligonucleotide Synthesis

1.2.3.1 Phosphodiester Method

The classical synthetic method, the phosphodiester approach, was pioneered by Khorana and colleagues.[16,17] The method involves the coupling of a 5'-protected deoxynucleoside with a 3'-protected deoxynucleoside 5'-phosphomonoester (***Figure 1.5***). The condensing agent, initially dicyclohexylcarbodiimide (DCC)[18] but more recently triisopropylbenzenesulphonyl chloride (TPSCl),[19] activates the phosphomonoester. The 3'-protecting group of choice is commonly acetyl or a silicon derivative and is therefore removed by alkali or fluoride ions respectively, allowing a 5' to 3' chain extension. The 5'-protecting group used by Khorana was 4-monomethoxytrityl (R = MMTr).

However, a major drawback in this method is the susceptibility of the unprotected phosphate group in the product to further attack by activated phosphomonoester. An aqueous work-up is required to partly reverse this process but poor reaction yields, lengthy coupling times and extensive purification requirements result. Thus the synthesis of long oligonucleotides is an extremely time consuming and low yielding procedure.

The coupling of the 5'-hydroxyl group of a nucleoside to a solid support (***Figure 1.5***, R = solid support) led to some improvements but the fundamental problem remained.

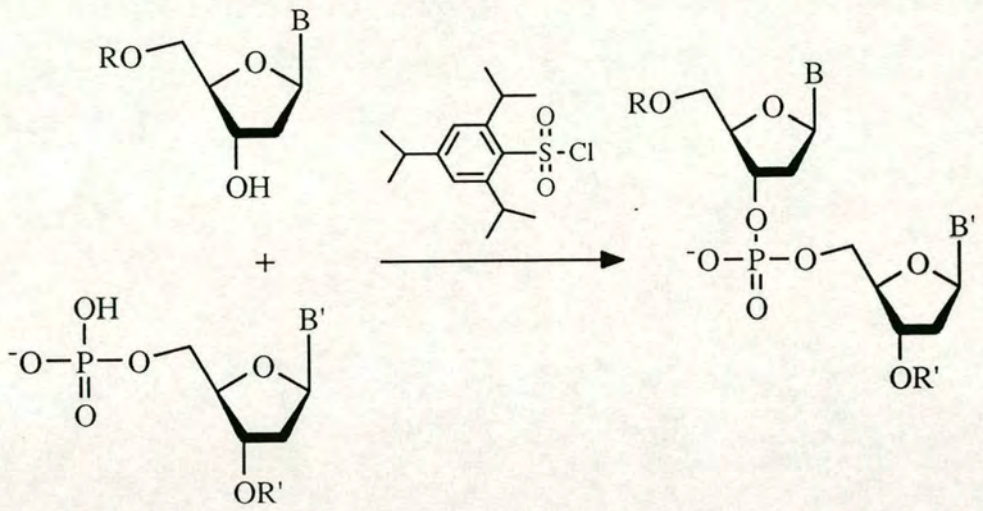


Figure 1.5 : The Phosphosdiester Approach

1.2.3.2 Phosphotriester Method

The phosphotriester approach[20] was investigated initially in the 1950s[2] but it was not until the early 1970s[21,22] when the process was further developed that it began to supercede the phosphodiester method. The weakness of the phosphodiester coupling is avoided by the introduction of a phosphate protecting group. Initial protecting groups such as β-cyanoethyl[11,23] and 2,2,2-trichloroethyl,[24] have since been replaced by aryl groups, the 2-chlorophenyl group in particular.[25-28] The aryl esters can be removed by treatment with certain oximes[29] or prolonged treatment with ammonium hydroxide.[30] The condensing agent has similarly developed from arylsulphonyltriazoles[31] and arylsulphonyltetrazoles[32] to the current agent of choice, 1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole (MSNT).[33] MSNT activates the 3'-deoxynucleoside phosphodiester in the coupling reaction (***Figure 1.6***). The 2 and 6 methyl groups of the mesitylene ring provide steric hindrance at the sulphur to ensure condensation at phosphorus. Often N-methylimidazole is added to enhance the reaction rate,[12] reducing the standard coupling cycle to as little as 15 minutes.

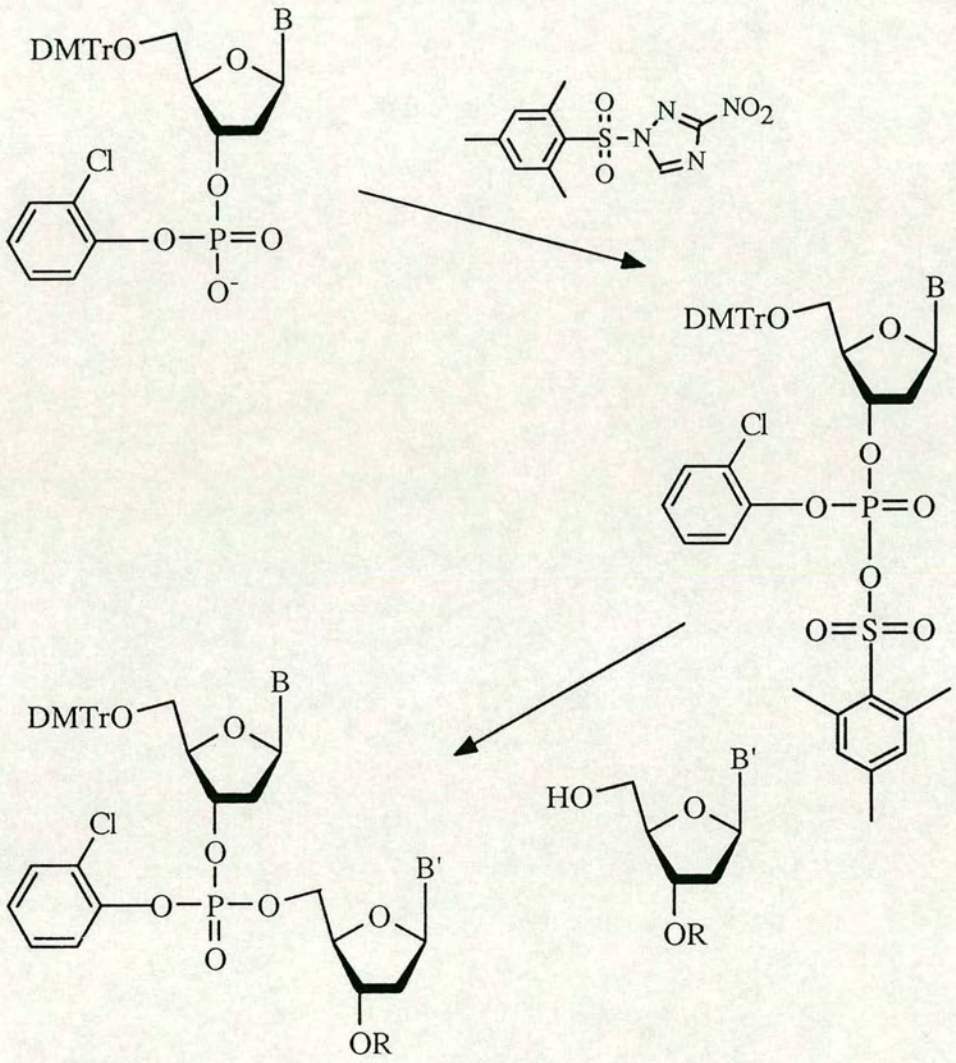


Figure 1.6 : The Phosphotriester Approach

It acts as a nucleophilic catalyst, forming an N-methylimidazolium phosphodiester with the N-methylimidazole a superior leaving group. The coupling reaction is normally carried out in pyridine with the triethylammonium salt of the nucleotide monomer. Chain extension is in the 3' to 5' direction.

The coupling yields are limited to 97-98% due to a competing 5'-hydroxyl sulphonation reaction by the MSNT. The vulnerability of the O-6 position of deoxyguanosine and the O-4 position of thymidine to nitrotriazole substitution leads to a further side reaction unless these groups are protected. Thus oligonucleotide length is restricted to 40-50 residues.

Solid phase synthesis was successfully carried out using phosphotriester chemistry[34] (***Figure 1.6***, R = solid support) but this has largely been superceded by phosphite triester solid phase synthesis due to the superior coupling yields and faster coupling times of the latter. However, phosphotriester chemistry does have its advantages: the stability and ease of handling of the monomers; the removal of small quantities of moisture in the coupling step by MSNT; the need for only slight excess of monomer; and the viability of the method for solution phase synthesis: the latter two being particularly advantageous for large scale synthesis.

1.2.3.3 Phosphite Triester Method

The value of trivalent phosphorus in oligonucleotide synthesis was first realized by Letsinger,[35] using chlorophosphites, but it was the introduction of the more stable phosphoramidites by Caruthers[36] which brought the real breakthrough. The initial dimethylamino groups were replaced by others[37] such as N-morpholino and, in particular, N,N-diisopropyl. The advent of solid phase synthesis has made the phosphite triester method particularly advantageous.[38]

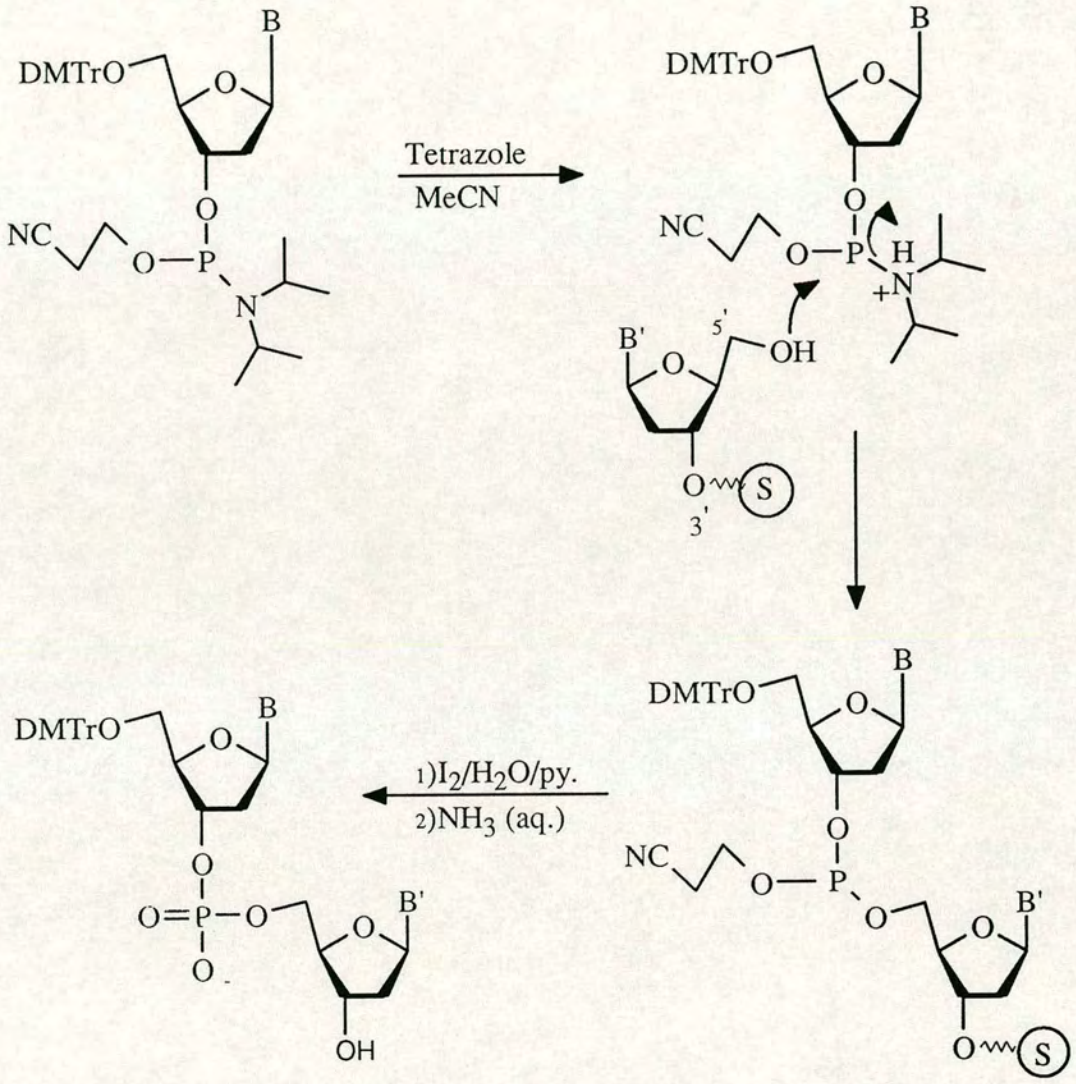


Figure 1.7 : The Phosphite Triester Approach

The key step in the method is the highly efficient coupling between the free 5'-hydroxyl group of the support bound oligonucleotide and the 5'-protected phosphoramidite monomer in solution (***Figure 1.7***). The nitrogen atom of the phosphoramidite group is protonated by a weak acid (tetrazole) which leaves the DMTr group untouched. Coupling is followed immediately by a capping step using acetic anhydride and N-methylimidazole to block any unreacted 5'-hydroxyl groups in order to simplify purification. The capping step also reverses any undesirable phosphitylation of the O-6 position of guanine. Iodine oxidation produces the phosphotriester and chain extension can continue. Each synthesis cycle can take as little as 5 minutes. Once oligonucleotide synthesis is complete and solid support cleavage has occurred, the phosphate protecting group of choice, β-cyanoethyl, is removed with ammonium hydroxide treatment at the same time as removal of the base protecting groups.

As a consequence of the high coupling yields (>98%) and rapid coupling times, oligonucleotides of up to 100 residues in length can be made with ease. The only minor drawback is the susceptibility of P(III) reagents to oxidation and hydrolysis on storage.

1.2.3.4 H-Phosphonate Method

As with the phosphotriester method, H-phosphonate chemistry has its origins with Todd in the 1950s[39] but has only recently resurfaced[40,41] as a viable method for oligonucleotide synthesis. Again, it is usually employed on a solid support with a 3' to 5' chain extension (***Figure 1.8***). The coupling reaction is activated by a hindered acyl chloride such as pivaloyl chloride (PVCl) and the resultant H-phosphonate diester is relatively stable both to further coupling and to acid catalysed cleavage. Hence no phosphate protecting groups are required and iodine oxidation only occurs once as the penultimate step which is followed by solid support cleavage.

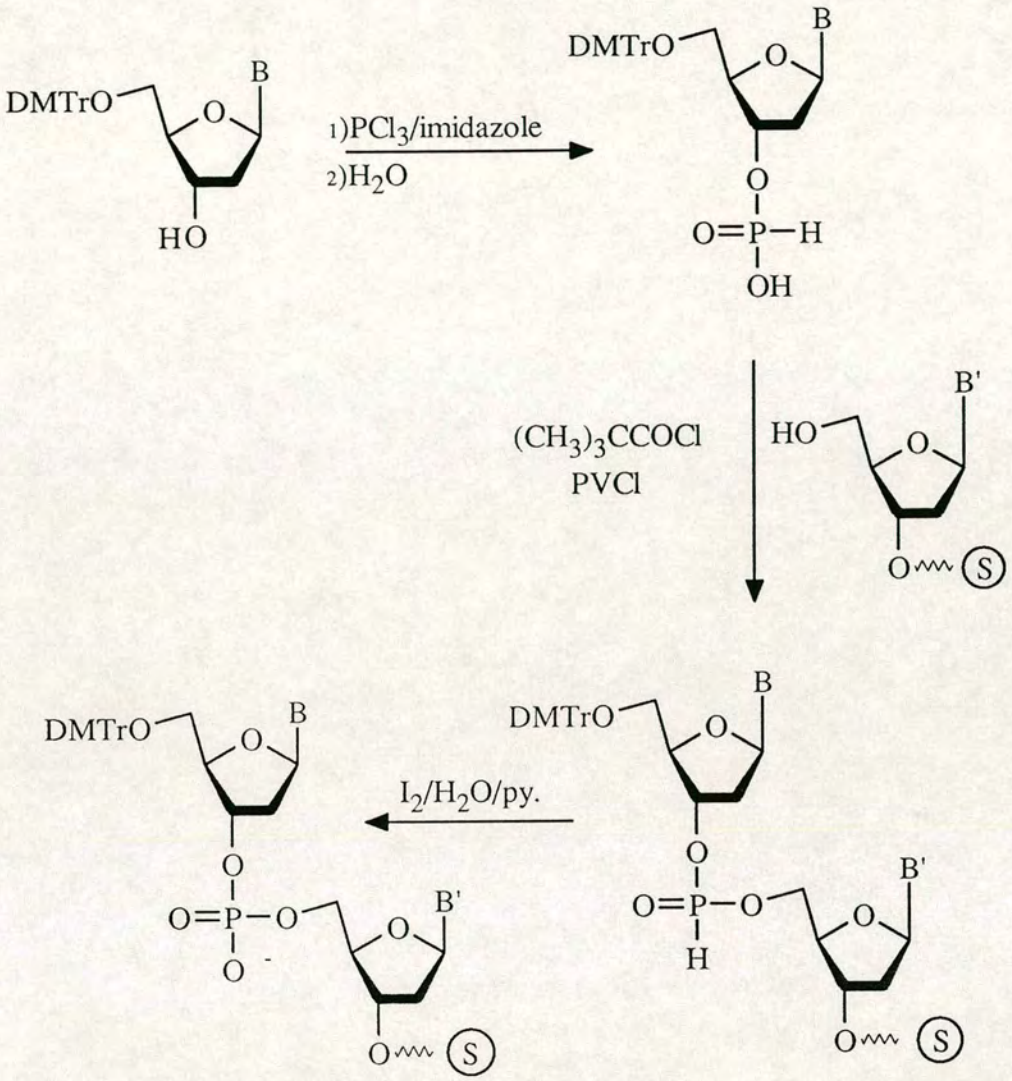


Figure 1.8 : The H-Phosphonate Approach

In addition, coupling is rapid and efficient and the rigorously anhydrous conditions necessary in the phosphoramidite approach are not essential. However, the coupling efficiency is not as high as in the phosphoramidite approach due to a side reaction; in the presence of the activating agent, the H-phosphonate monomer tends to dimerise to form a symmetrical phosphite anhydride.

1.3 Antisense Oligonucleotides

1.3.1 The Antisense Theory

As protein synthesis in cells is the result of two basic steps, transcription and translation, the basic antisense method[42,43] to inhibit protein synthesis is straightforward: introduce a synthetic DNA molecule of base sequence complementary to that of the mRNA to be inhibited. The ribozomes are prevented from reading the genetic code from the mRNA:DNA hybrid duplex and the action of RNaseH,[44] an RNA digesting enzyme involved in DNA replication but also present in the cytoplasm, digests the mRNA. It is known that antisense regulation of gene expression exists as a naturally occurring mechanism in cells.[45]

An immediate application of this theory is the selective inhibition of viral protein synthesis. Given the DNA sequence of a particular virus, readily obtainable using standard procedures, an oligonucleotide drug molecule can be designed. If an accessible region of the viral gene is chosen and the oligonucleotide is of sufficient length to ensure selectivity, it should have enormous advantages over small molecule drugs.

Particular attention has been focused on retroviruses such as the human immunodeficiency virus (HIV),[46] the retrovirus responsible for acquired immune deficiency syndrome (AIDS). Anti-HIV treatment to date has focused on 3'-azido-2',3'-dideoxythymidine (AZT)[47] and other dideoxynucleosides which operate as reverse transcriptase inhibitors and chain terminators. However, they are unsatisfactory antiviral agents due to toxicity and the resistance of certain viral strains.

Retroviruses enter the host cell in RNA form and the RNA is copied into viral DNA by the action of the viral DNA polymerase, reverse transcriptase. The viral DNA molecules are integrated into the nuclear DNA of the host cell and normal cellular transcription and translation lead to the synthesis of viral protein and the virus

proliferates. The final step in this process is the target for antisense oligonucleotides (***Figure 1.9***).

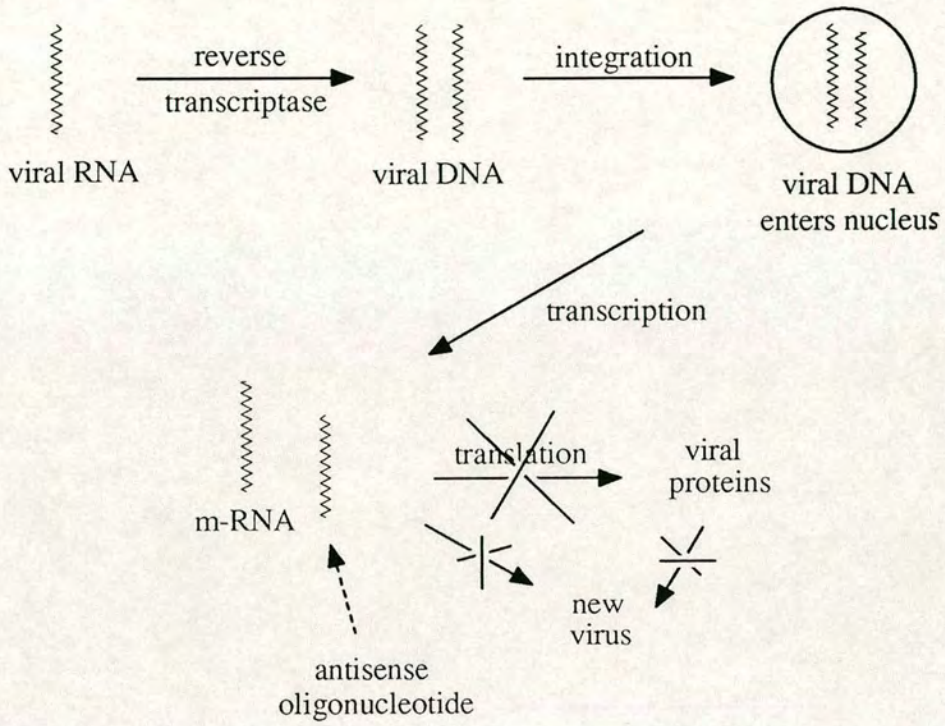


Figure 1.9 : Antisense Viral Inhibition

Early work in this field[48,49] was carried out on the Rous sarcoma virus using complementary 13mer (13 base pairs in length) oligonucleotides. Despite the strictly limited success of unmodified oligonucleotides,[50-53] the antisense theory was validated. Problems arose largely due to the instability of oligonucleotides in cells. Single stranded DNA is degraded by DNase enzymes present in serum and cytoplasm.[54] In order to prevent this, chemically modified oligonucleotides are now used.

1.3.2 Modified Synthetic Antisense Oligonucleotides

Clearly, any modification introduced into the antisense DNA molecule must not greatly reduce the stability of the DNA:RNA hybrid duplex which is the substrate for RNaseH.[55,56] The molecule must also possess low toxicity.

1.3.2.1 Phosphorothioates

Probably the most widely used of all DNA analogues are phosphorothioate oligonucleotides in which one of the non-bridging oxygen atoms in each internucleotide phosphate linkage is replaced by a sulphur atom. Although some depression of melting temperature occurs,[57,58] considerably improved nuclease resistance is observed and potent antisense effects in HIV[59-62] and other systems[63-65] have been recorded. However, activity was initially only observed in *de novo* systems[59] and the unexpectedly high activity of an oligo dC phosphorothioate (SdC28) revealed a second, non-sequence dependent mode of inhibition which was shown to arise from interaction of the oligonucleotide with HIV reverse transcriptase.[66]

Despite the complexities of HIV as a retrovirus, two regulatory genes, *tat* and *rev* (*art/trs*),[67] both thought to be essential for viral replication, have been identified. Clearly, inhibition of either of the genes would inhibit virus assembly. A 28mer phosphorothioate, SRev d(TCG TCG CTG TCT CCG CTT CTT CCT GCC A), complementary to a region from the 2nd to the 29th bases of the *rev* gene showed significant inhibition of *rev* protein synthesis in chronically infected cells.[61] This led to the drastic reduction of unspliced viral mRNA transcripts since the *rev* gene regulates the expression of viral proteins by influencing the splicing of the viral mRNA. Other phosphorothioates of similar length targeted in the same region showed lesser degrees of HIV inhibition. The absence of significant activity using phosphorothioate 28mer of random sequence and an antisense sequence with unmodified normal

phosphodiester linkages showed that sequence specificity and nuclease resistance are essential for viral inhibition.

1.3.2.2 Phosphorothioate Oligonucleotide Synthesis

The synthesis of phosphorothioate oligonucleotides is most commonly achieved in the phosphoramidite method by replacing the oxidation step in each synthesis cycle by a sulphurisation step. This is a matter of simply replacing the iodine/pyridine bottle on the automated DNA synthesiser by the sulphurisation reagent and allowing a longer reaction time. Tetraethylthiuram disulphide (TETD)[68] has now replaced elemental sulphur in carbon disulphide as the reagent of choice, achieving sulphurisation in comparable efficiency to iodine oxidation in a 15 minute step (***Figure 1.10***). The undesirable Arbuzov side reaction (B), caused by dithiocarbamate attack on the cyanoethyl group is blocked by the steric bulk of either the phosphite or thiuram substituents. The desired pathway (A) predominates almost exclusively.

Phosphorothioates can also be synthesised using phosphotriester chemistry. However, the process is somewhat more laborious because the coupling reaction analogous to the standard phosphotriester chemistry using 2-chlorophenylphosphorodichloridothioate[69,70] as the phosphorylating reagent does not work.[71] A leaving group superior to the chloride anion is required for the coupling step and attention has focused on 1-hydroxybenzotriazole[72] and analogues,[73] the most successful of these being the 1-hydroxy-6-trifluoromethylbenzotriazoyl group (***Figure 1.11***).[74,75]

The H-phosphonate method is employed to make complete phosphorothioates with the advantage of requiring only a single sulphurisation step at the end of the solid phase oligonucleotide synthesis. However, it cannot be used to make mixed phosphorothioate/phosphodiester oligonucleotides because phosphorothioate diesters are converted by iodine/water to phosphodiesters.

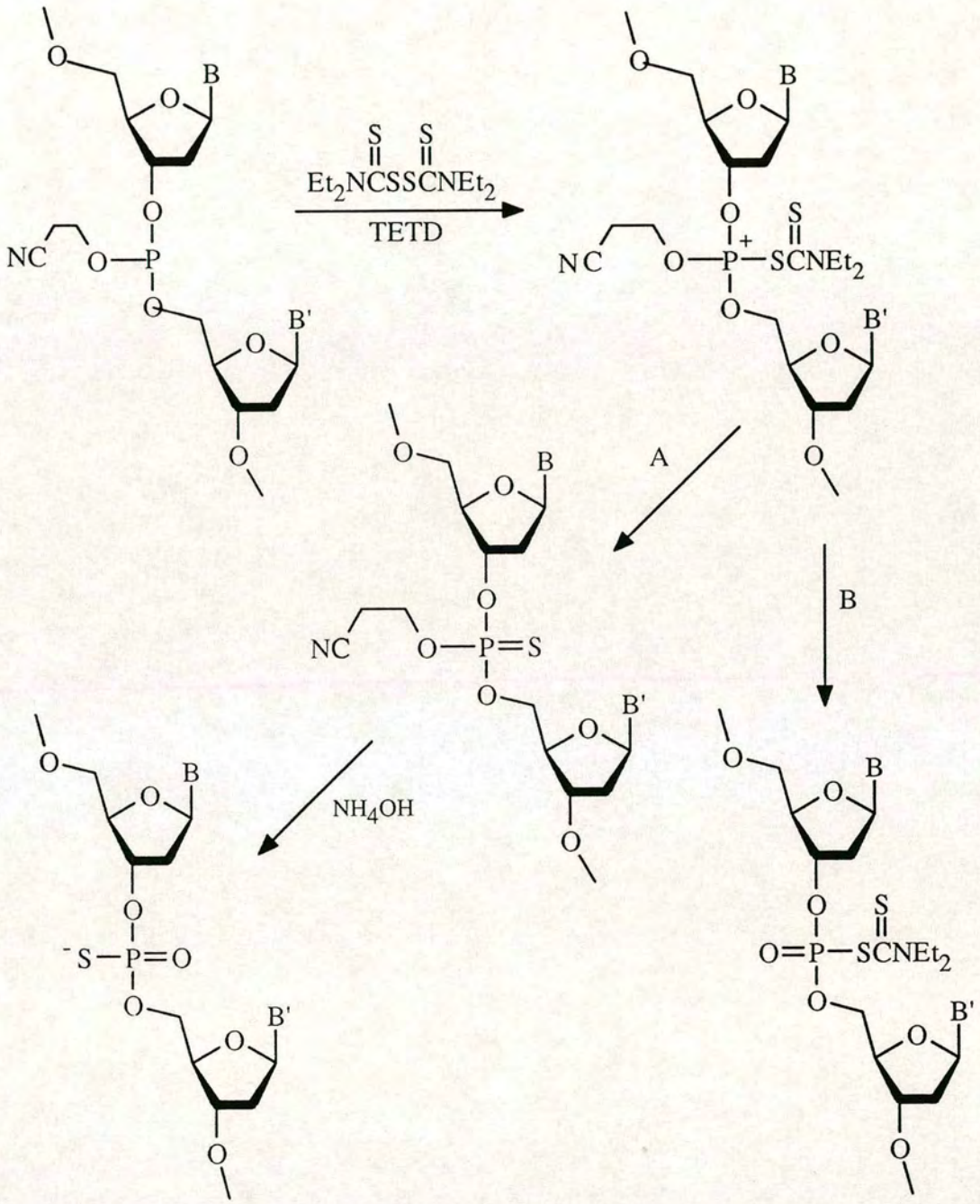


Figure 1.10 : Sulphurisation of the Phosphoramidite Group During Oligonucleotide Synthesis
A: Desired Reaction B : Arbuzov Side Reaction

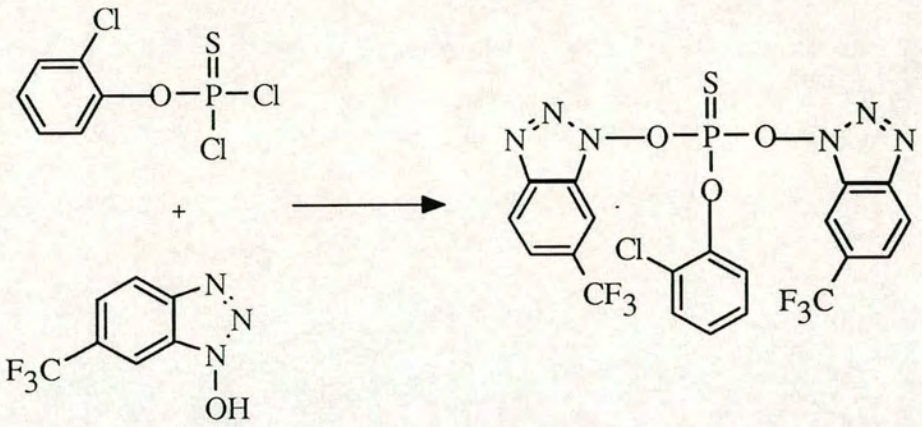


Figure 1.11 : The Synthesis of the Phosphorylating Reagent in the Phosphotriester route to Phosphorothioates

In all the above methods, a mixture of R and S enantiomers at the phosphorus atom is formed (***Figure 1.12***) and efficient, reliable processes for the synthesis of chirally pure phosphorothioates are still being sought.[76]

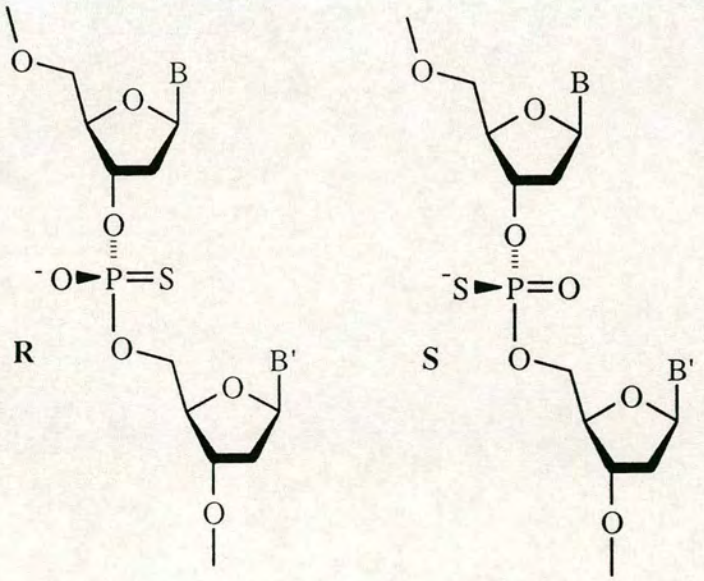


Figure 1.12 : Phosphorothioate Stereochemistry

1.3.2.3 Other Backbone Modifications

Phosphorodithioates (***Figure 1.13***),[77] where both non-bridging oxygen atoms in the phosphate linkage are replaced by sulphur, possess an achiral phosphorus centre. However, the antisense activity of these compounds[78] appeared to be less promising than for the phosphorothioate analogues.

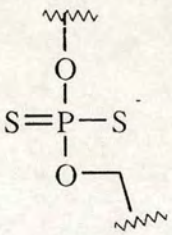
Oligonucleotide methylphosphonates (***Figure 1.13***),[79] with a phosphate oxygen atom replaced by a methyl group, were among those early analogues which heralded the recent rapid expansion in the numbers of backbone modified oligonucleotides. They have an uncharged backbone and therefore have increased hydrophobicity leading to improved cell membrane permeabilility properties, nuclease stablility and duplex stability relative to phosphorothioates.[58] They have been tested for activity against HIV,[80] herpes simplex virus I (HSVI)[81] and other viruses.[63,82] However, the chirality problem still exists and as hybrids of these analogues with RNA are not substrates for RNaseH,[55,56] unlike phosphorothioates, antisense effects are reduced. In addition these analogues have poor aqueous solubility.

5'-Amino nucleosides have long been known[83] and phosphoramidate oligonucleotides (***Figure 1.13***), with a bridging phosphate oxygen atom replaced by nitrogen, have been known for almost as long.[84] However, with the advent of the antisense method, superior synthetic procedures have been developed.[85,86] These analogues have an additional property: selective acidic cleavage of the oligonucleotide at the P-N bond is possible. Early hybridisation work[87] suggested an increase in melting temperature for an oligonucleotide with a single 3'-terminal amino group, although 5'-terminal and internal phosphoramidates appear to destabilise the duplex.

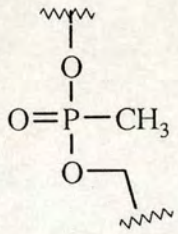
Enzymatic oligonucleotide degradation in cells appears to be mostly due to 3' to 5' exonucleases although endonucleases and 5' to 3' exonucleases are also present. Thus nuclease stabilty can be enhanced simply by introducing a modification at the 3' end of the oligonucleotide.[88] Examples of this are intercalators such as acridine (***Figure 1.13***)[89] which also facilitates cellular uptake and provides additional binding

energy by inserting (intercalating) between the base pairs of the oligonucleotide and its complementary sequence. It has also been suggested that antiviral activity of intercalator bound oligonucleotides can occur as a result of triple-helix formation between the antisense strand and viral DNA.[90]

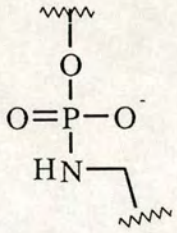
Another class of synthetic antisense oligonucleotide is α-anomeric DNA (***Figure 1.13***) in which the β-linkage between the nucleotide sugar and base is replaced by the unnatural α-linkage. Nuclease stability is greatly increased and in certain sequences these analogues form hybrids with RNA which are more stable than the β-oligonucleotides.[91] Unfortunately they are extremely expensive to synthesise. Attempts have been made to combine them with intercalators.[92]



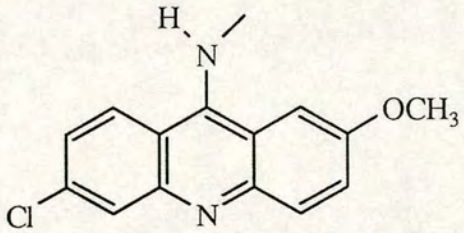
Phosphorodithioates



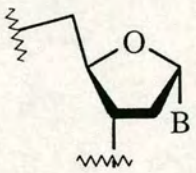
Methyl Phosphonates



Phosphoramidates



Acridine



α-anomeric

Figure 1.13 : Examples of Backbone Modifications

Nuclease stability may also be conferred on an oligonucleotide by introducing modified bases, pyrimidines in particular,[93] without adversely affecting duplex stability. It is claimed that modifications at the 5 or 6 positions of cytidine or thymidine block a nuclease binding site. In an attempt to achieve irreversible inactivation of the target oligonucleotide by cross-linking across the major groove, an alkylating electrophile, the 3-(iodoacetomido)propyl group, was joined to the 5 position of a thymidine.[94]

Alterations to the sugar ring include the substitution of hexose for pentose sugars[95] and a 4'-thio group to replace the sugar bridging oxygen.[96] Other more radical backbone modifications involve completely replacing the phosphate internucleotide linkage. Examples of novel linkages include the methylhydroxylamine,[97] carboximide,[98] formacetal[99] and guanidine[100] groups. In addition to being nuclease resistant, oligonucleotides containing these modifications show surprisingly little depression of melting temperature.

An interesting recent development has been to combine peptide-like backbones with nucleic acid bases. The idea originally appeared some years ago[101] but it has attracted fresh interest due to the extremely high duplex stability discovered for a particular peptide nucleic acid analogue.[102] Melting temperatures are considerably higher than the equivalent unmodified hybrids although questions have arisen over the sequence specificity of such molecules.

1.3.3 Oligonucleotides Linked to Transport Agents

Oligonucleotides are large, polyanionic molecules which therefore have poor cell permeability properties although the exact nature of oligonucleotide/phospholipid membrane interactions is unclear.[103] In the case of the phosphorothioate oligonucleotide, SRev, the dose required to achieve significant anti-HIV effects is quite high (~25μM). By linking the antisense oligonucleotide to transport agents,

largely bulky, lipophilic molecules, uptake and thus antiviral activity might be enhanced.

Cholesterol covalently linked to oligonucleotides has been the most widely investigated transport agent. The coupling reaction has been performed using phosphoramidite cholesterol monomers[104,105] or using H-phosphonate chemistry[106,107] or post-synthetically via a urethane linker.[105] Having a 3'-terminal cholesterol in order to engender greater nuclease stability,[88] has been achieved via a carboxyaminoethyl linker,[108] coupling to the N-6 position of a 3'-adenosine,[109] post synthetically[110] or through attachments to the solid support via a variety of different groups: solketal,[104] hydroxy-L-proline,[111] pyrrolidinylbutanedioate[88] or aminoglycerol.[105] Cholesterol can be linked to an oligonucleotide via a disulphide group using H-phosphonate chemistry[112] or by a disulphide exchange reaction.[113,114]

The coupling of a cholesterol phosphoramidite directly to SRev presents a new problem.[104] The normal cholesterol phosphoramidite coupling produces a phosphite diester linkage between the cholesterol group and SRev; sulphurisation of which with TETD failed due to extreme steric hindrance. By reacting cholesteryl chloroformate **[1]** with 6-aminohexan-1-ol (***Figure 1.14***), a carbamate linked hexyl spacer with an available hydroxyl group **[2]** was synthesised. The resulting unhindered monomer **[3]**, when coupled to SRev, sulphurised smoothly.

Long, alkyl chains have been covalently linked to oligonucleotides. The 1,2-di-O-hexadecylglyceryl group has been synthesised[115] from allyl alcohol (***Figure 1.15***). Tetrahydropyranyl (THP) protection of the hydroxyl was followed by oxidation with potassium permanganate.[116] Subsequent reaction with hexadecyl mesylate[117] and deprotection[118] led to the product **[4]**. This was coupled to the oligonucleotide 5'-terminus using phosphoramidite[104] or H-phosphonate[119] chemistry.

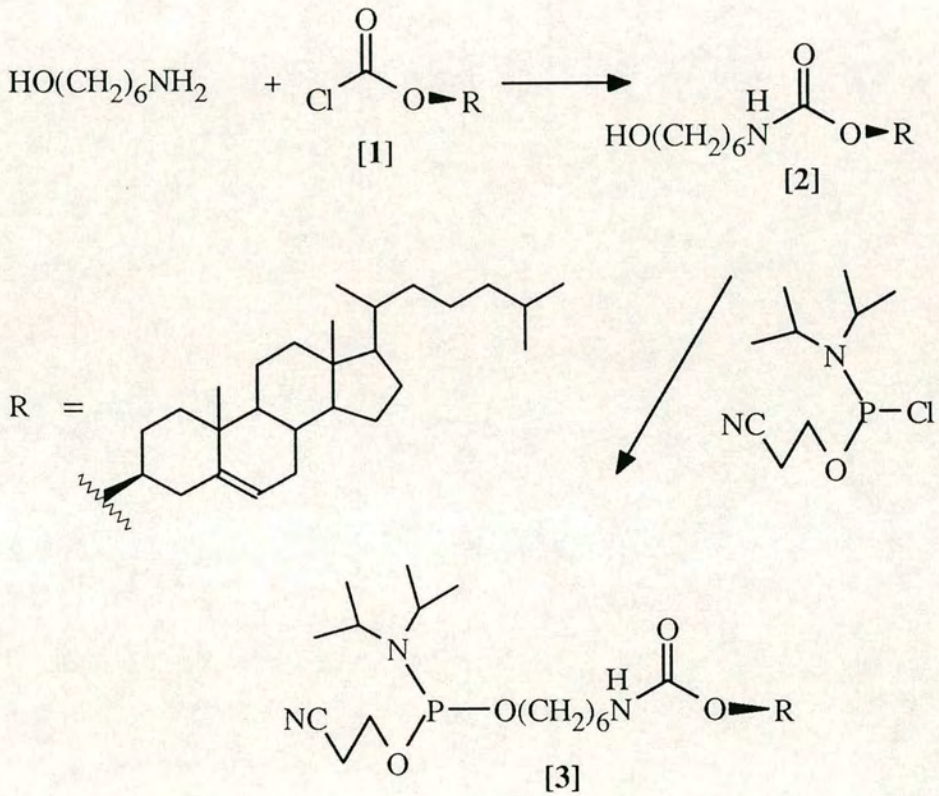
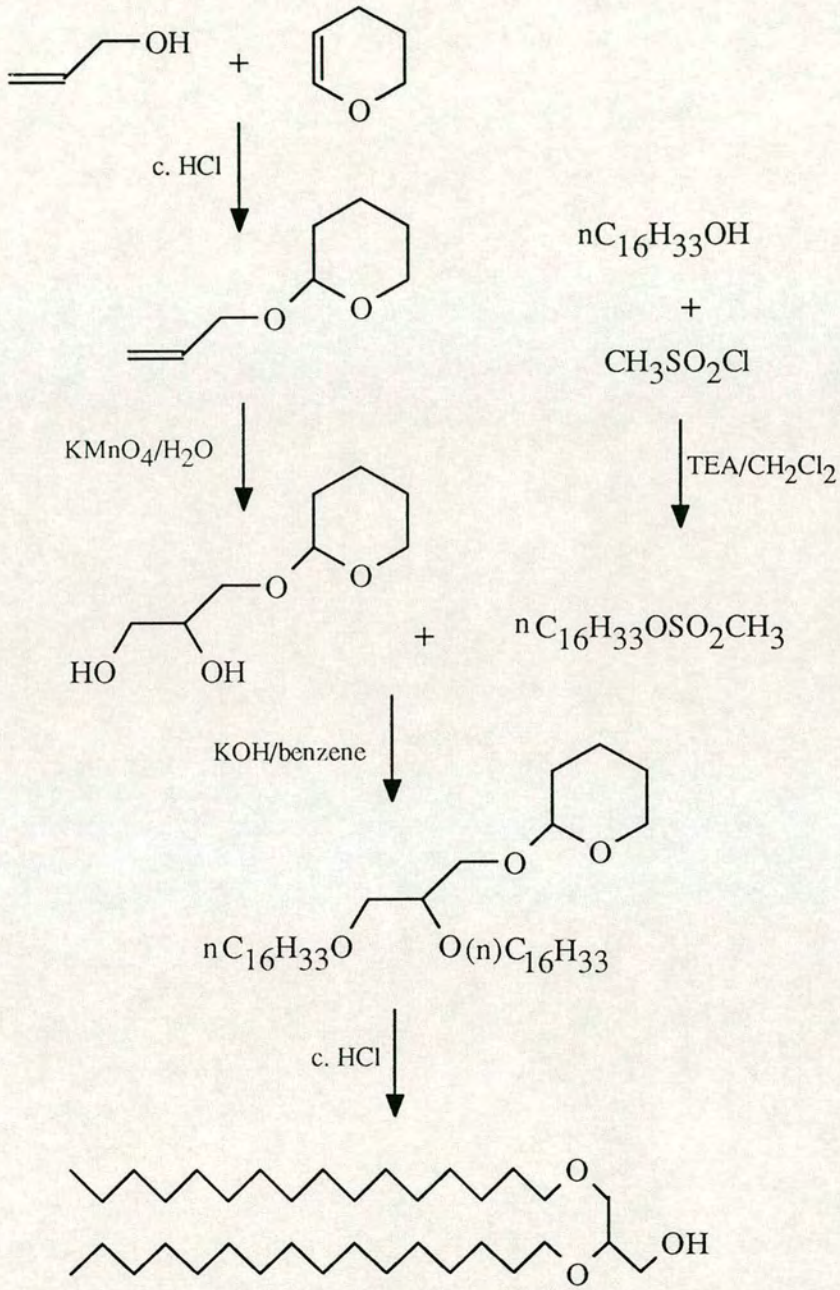

HO(CH2)6NH2
+
Cl
O
O
R
[1]
HO(CH2)6N
H
O
O
R
[2]
R =
N
P
Cl
NC
O
N
P
O(CH2)6N
H
O
O
R
NC
O
[3]

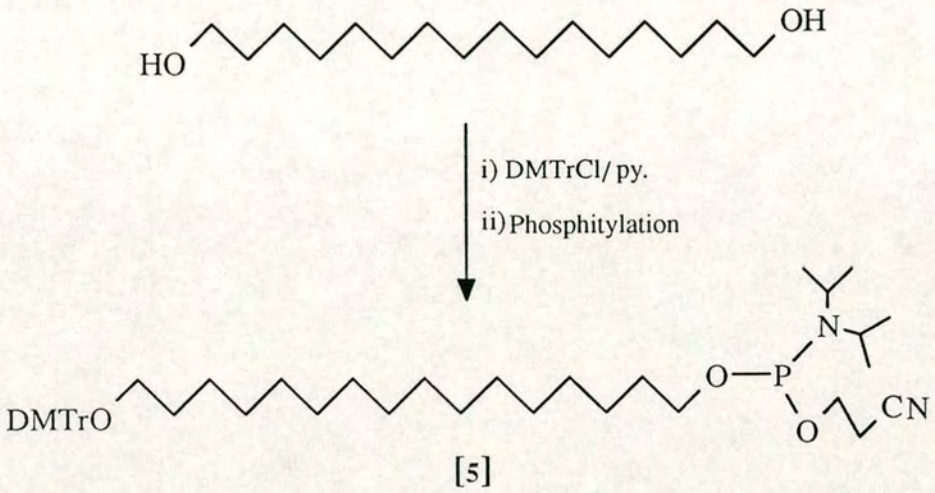
Figure 1.14


OH
+
O
c. HCl
O
O
$nC_{16}H_{33}OH$
+
CH_3SO_2Cl
$KMnO_4/H_2O$
TEA/CH_2Cl_2
O
O
HO
OH
+
$nC_{16}H_{33}OSO_2CH_3$
KOH/benzene
O
O
$nC_{16}H_{33}O$
$O(n)C_{16}H_{33}$
c. HCl
O
OH
O

[4]

Figure 1.15

The hexadecyl phosphoramidite monomer **[5]** (***Figure 1.16***) was coupled to both 5'- and 3'-termini of different oligonucleotides.[104] Similarly, 5'-octadecyl[104] and undecyl[120] and 3'-dodecyl[121] groups were bonded to antisense oligonucleotides.



[5]

Figure 1.16

Other less common groups used as transport agents include poly-L-lysine,[122] vitamin E,[123] adamantyl[104] and phenazinium.[110]

In some assay systems, lipophilic modified oligonucleotides show increased antiviral activity and this is assumed to be the direct result of cellular uptake changes,[106-108,112-114] although the effect seems to be non-sequence dependent in several cases.[106,107,112]

Although oligonucleotide duplex stability has been reported to be adversely influenced by the presence of a terminal lipophilic group,[108,112] or not influenced much at all,[104] it has more generally been the case that the effects are favourable.[88,105,111,113] More than one cholesterol group in a single oligonucleotide engenders notable extra stability in both duplexes and triplexes,[105] presumbly through inter-cholesterol hydrophobic interactions.

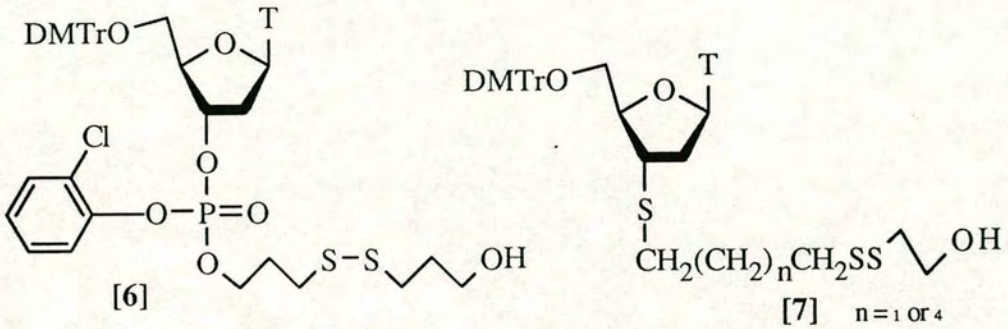
1.4 Functional Groups in Oligonucleotides

1.4.1 Disulphides in Oligonucleotides

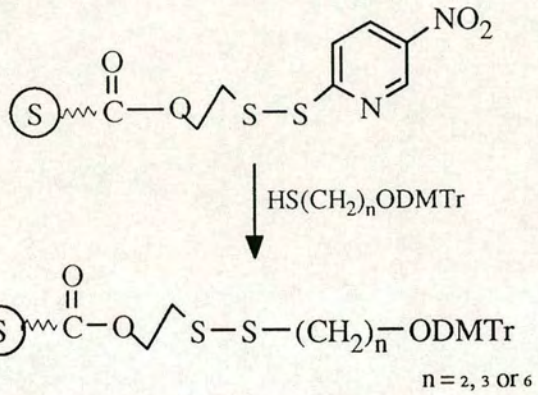
3'-Alkyl disulphides have been introduced into oligonucleotides using modified solid supports (***Figure 1.17***).[124-127] Zuckerman *et al*[124] phosphorylated a 5'-protected nucleoside at the 3'-position then introduced the 3,3'-dithiodipropanol group **[6]** or alternatively, for thymidine, reacted 2,3'-anhydrothymidine with either 1,3-propanedithiol or 1,6-hexanedithiol then coupled with S-(2-thiopyridyl)-2-mercaptoethanol to introduce the disulphide **[7]**. More simply, Gupta *et al*,[125] initially coupled S-(2-thio-5-nitropyridyl)-2-mercaptoethanol onto the solid support, then, by a disulphide exchange reaction, introduced the 4,4'-dimethoxytritylated n-mercaptoalkane of length C2, C3 or C6. The most facile approach was that of Asseline *et al*[126] in which 1-(4,4'-dimethoxytrityl)-2,2'-dithiodiethanol **[8]** was introduced directly onto the Fractosil 500 solid support, in development of the octaethyleneglycol work of Bonfils and Thuong.[127] In all cases, detritylation allowed automated oligonucleotide synthesis with every subsequent nucleotide coupling with standard efficiency.

Cleavage of disulphides is routinely carried out with an excess of dithiothreitol (DTT)[128] above pH7. For the C3 and C6 alkyl disulphide linkers, a 3'-thiol group was obtained. In the case of the C2 linker, however, DTT treatment led to a 3'-phosphate by elimination of mercaptoethanol (***Figure 1.18***).[125,126] Asseline *et al*[126] extended this idea to make 3'-terminal phosphorothioates and aminoalkyl derivatives.

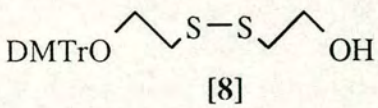
A 3'-disulphide group has also been introduced post-synthetically by reacting an oligonucleotide bearing a 3'-amino functionality on a modified cytidine residue, with dithio-bis-propionyl-N-hydroxysuccinimide **[9]**[129] or by reacting N-acetyl-N-(*p*-glycoxylbenzoyl)cystamine **[10]** with a 3'-deprotected guanosine residue on the oligonucleotide (***Figure 1.19***).[130] Disulphide cleavage and a disulphide exchange reaction with a disulphide derivatised protein led to a DNA/protein coupling.[130]



Zuckerman et al [124] modified 3'-nucleosides



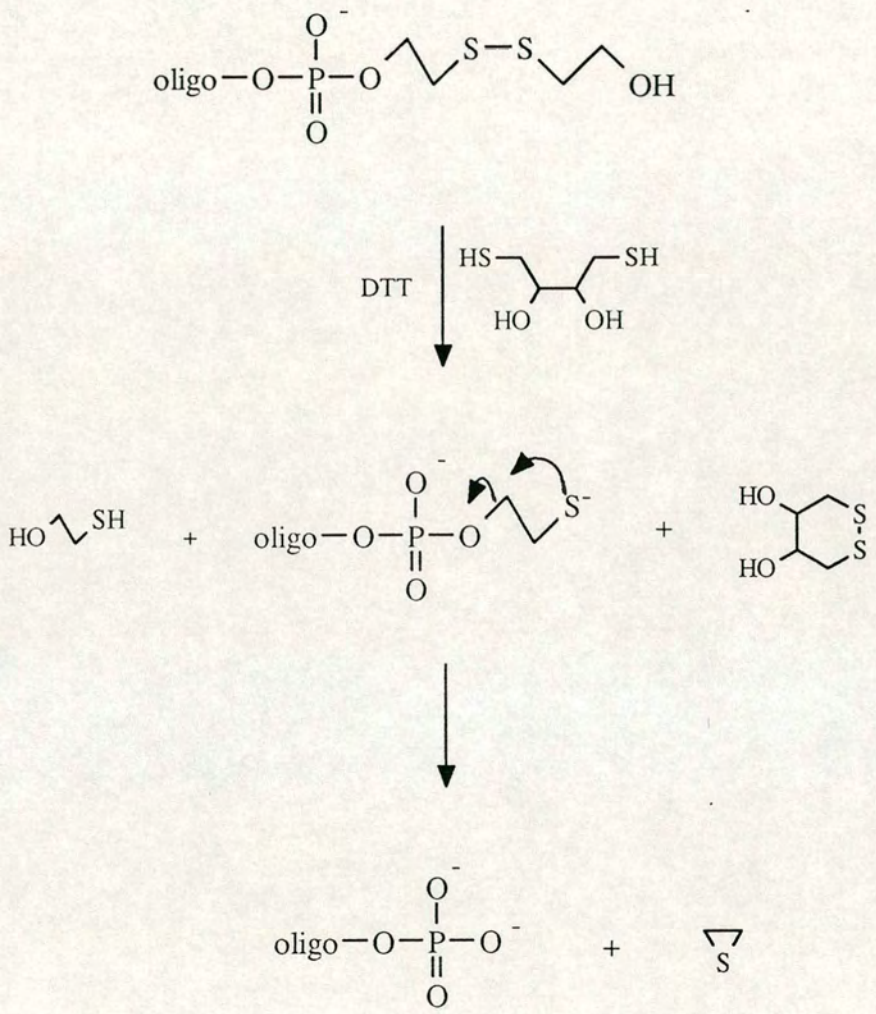
Gupta et al [125] modified solid support



[8]

Asseline et al [126] disulphide unit

Figure 1.17


oligo—O—P—O
O
O
S—S
OH
DTT
HS
SH
HO
OH
HO
SH
+
oligo—O—P—O
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HO
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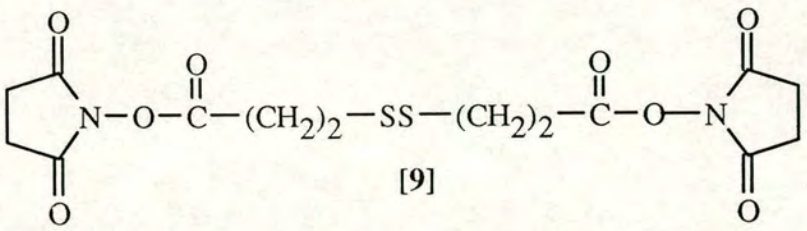
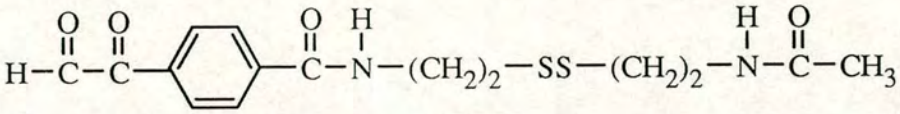
S
oligo—O—P—O
O
O
+
S

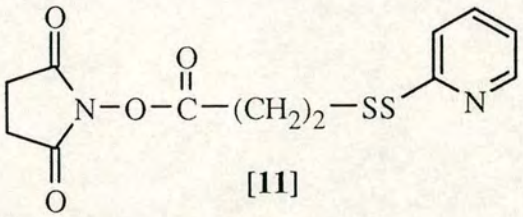
Figure 1.18



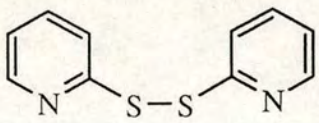
[9]



[10]



[11]

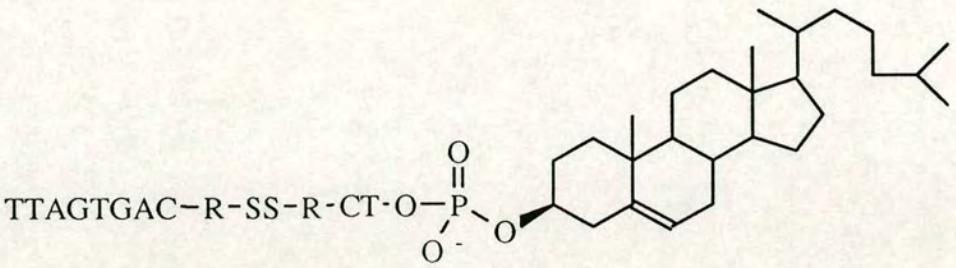


[12]

Figure 1.19

Oligonucleotides containing 5'-disulphides are less common. By reacting a 5'-amino functionalised oligonucleotide with N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) **[11]** (***Figure 1.19***), a 5'-disulphide hence a 5'-thiol was obtained.[131] The reaction of 3-thiocholesterol with dipyridyl disulphide **[12]** (***Figure 1.19***) gave a cholesterol disulphide which, by a disulphide exchange reaction with a cystamine oligonucleotide derivative, led to a cholesterol-disulphide oligonucleotide.[113] This has also been achieved more simply on solid phase via H-phosphonate chemistry in which 2-(4,4'-dimethoxytrityl)-2-hydroxyethyl disulphide or mono[2-(4,4'-dimethoxytrityl)](hydroxyethoxy)ethyl disulphide hydrogen

phosphonate monomers were incorporated into the 5' position of a d(CAGTGATT) sequence. Subsequent coupling of dT and dC monomers and a cholesterol hydrogen-phosphonate monomer led to the target oligonucleotide (***Figure 1.20***).[112]



where R = CH_2CH_2 or $CH_2CH_2OCH_2CH_2$

Figure 1.20

A disulphide has been introduced between a biotin group and an oligonucleotide (***Figure 1.21***)[132] by modifying the 5 position of a 2'-deoxyuridine 5'-triphosphate **[13]**. Reaction with mercury (II) acetate introduced a mercury atom into the 5 position which was substituted with an allylamine group. This then reacted with sulphosuccinimidyl-2-(biotinamido)ethyl-1,3'-dithiopropionate (NHS-SS-Biotin) **[14]** to introduce the biotin. The modified nucleoside triphosphate **[15]** was then incorporated into an oligonucleotide by nick translation.

Oligonucleotide blocks have been combined through a disulphide formed between two terminal phosphorothioates,[133] a process which offers easy reversibility.

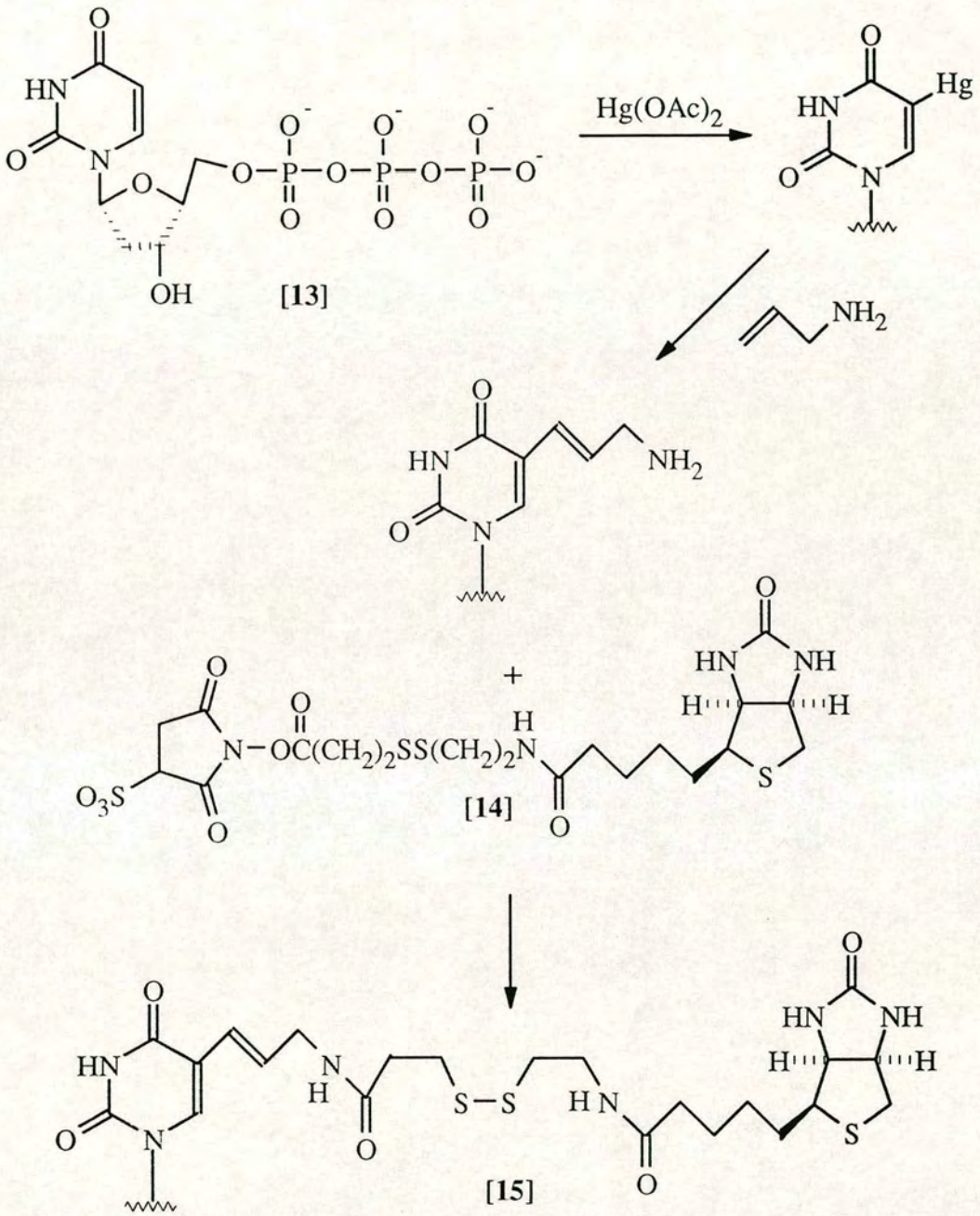


Figure 1.21 : Cleavable Biotin Monomer[132]

1.4.2 Terminal Thiol Groups

Disulphides are primarily introduced into oligonucleotides as a potential source of terminal thiols.[124-127,129,131] Direct solid phase routes to thiols (***Figure 1.22***) are limited in number. Connolly[134] coupled an alkyl linked tritylated thiol phosphoramidite monomer **[16]** at the 5'-end of an oligonucleotide. Sproat *et al*,[135] phosphitylated a 5'-tritylated thiol nucleoside **[17]** and coupled this to the 5'-end of an oligonucleotide. In both cases, cleavage of the trityl group was achieved with silver ions which were then removed by precipitation with DTT. Again, conversion of the C2 linked thiol group to the phosphate by elimination of mercaptoethanol was observed.[136] The process was extended to H-phosphonate chemistry with the standard oxidation step to form phosphodiester linkages. This was carried out before additon of the tritylated thiol H-phosphonate monomer which had to be oxidised under special conditions to avoid thiol loss.[137] Finally, the reaction of a 5'-amino functionalised oligonucleotide with a cysteine derivative led to an alkyl linked thiol.[138]

Reaction of a thiol with a suitable thiol specific reporter group is a straightforward means of confirming its identity. 5-Iodoacetamidofluorescein (IAF) **[18]** (***Figure 1.23***) has been used for such a purpose.[139]

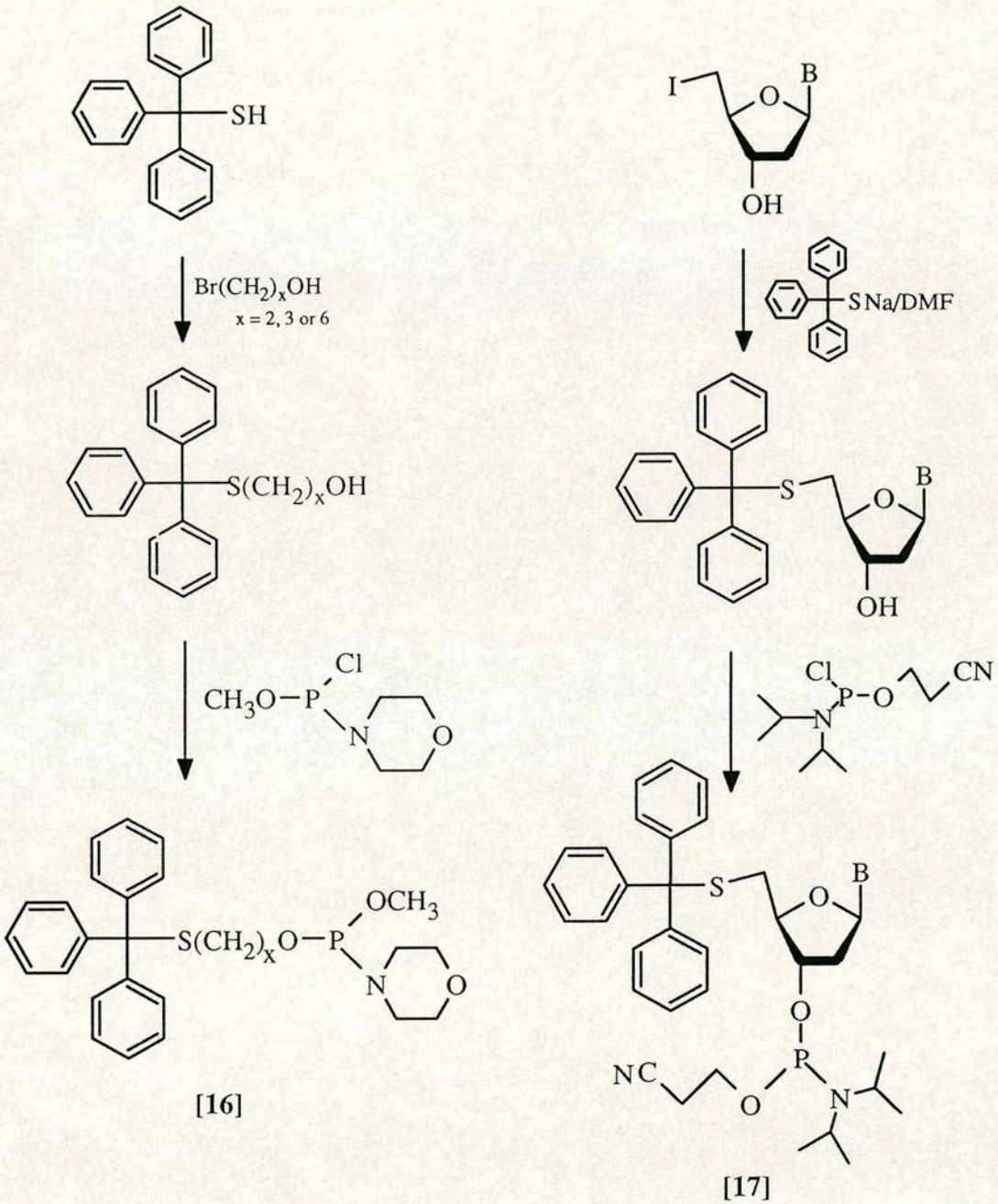
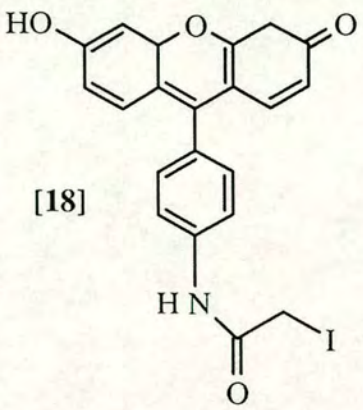


Figure 1.22 : Thiol Monomers[134,135]

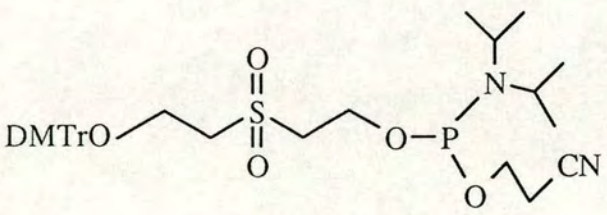


[18]

Figure 1.23 : 5-Iodoacetamidofluorescein

1.4.3 Terminal Phosphate Groups

5'-Terminal phosphate groups can be introduced enzymatically[140] but several procedures have been developed for their introduction during simple solid phase coupling reactions. To be compatible with phosphoramidite chemistry, a second phosphate protecting group is all that is required, which must be easily cleavable once oligonucleotide synthesis is complete. A second β-cyanoethyl group or alternatively a *p*-nitrophenylethyl group have been used both of which are cleaved by strong base after oligonucleotide purification.[141] However, the reagent of choice is the (2-cyanoethyl)-2-[2'-(4,4'-dimethoxytrityl)ethylsulphonyl)]ethoxy-N,N-diisopropylphosphoramidite monomer[142,143] **[19]** (***Figure 1.24***).



[19]

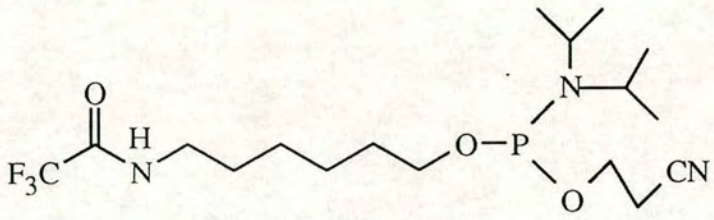
Figure 1.24: Terminal Phosphate Monomer[142,143]

It possesses a DMTr group to permit measurement of coupling efficiency and the phosphate protecting group is readily cleaved by base under standard oligonucleotide deprotection conditions. Furthermore, with β-elimination possible at either end of the molecule, it can be used to synthesise both 5'- and 3'-terminal phosphates and is superior to other solid support modification methods.[144,145] Coupling onto the 3'-support bound nucleotide allows chain extension through the other hydroxyl function present with final deprotection cleaving both protecting group and the 3'-nucleotide. Similarly, by replacing the oxidation step with a sulphurisation step, 3'- and 5'-terminal phosphorothioates can be synthesised. The only drawback to the monomer occurs during oligonucleotide purification. In reverse phase high performance liquid chromatography (HPLC), the standard method of oligonucleotide purification, the molecules elute in reverse order of lipophilicity. Without a hydrophobic group such as *p*-nitrophenylethyl,[141] it is difficult to separate the terminal phosphate oligonucleotide from the unphosphorylated failure sequences.

1.4.4 Terminal Amino Groups

The amino group is an extremely useful functional group for the terminus of an oligonucleotide. It can be reacted with electrophiles for the synthesis of further derivatives and is far more reactive than the normal terminal hydroxyl group. There are many methods for the attachment of such a functionality varying from post-synthetic addition by activation of the 5'-hydroxyl[146] or the 5'-phosphate[147] groups then addition of a diaminoalkyl reagent, solid phase triester chemistry using aminoalkyl monomers[148,149] or H-phosphonate chemistry.[137] However, the most useful method is direct attachment during phosphoramidite solid phase synthesis. In this way it is possible to add various protected aminoalkyl monomers,[137] in particular, the N-trifluoroacetyl-2-aminoethanol monomer in which the trifluoroacetyl protecting group is removed during extended ammonia treatment.[150] The extension of this to the aminohexanol equivalent, the 'aminolink 2' monomer **[20]** (***Figure 1.25***) is particularly

effective.[151] The 9-fluorenylmethoxycarbonyl (FMOC) protected aminoethanol analogue has also been made.[152]



[20]

Figure 1.25 : 'Aminolink 2' Monomer[151]

However, once the aminolink and subsequent derivatives have been attached to oligonucleotides, there is no easy way to reverse the process. A cleavable aminolink to regenerate the oligonucleotide would be extremely useful.

1.5 Biotin Labelled Oligonucleotides

Biotin (***Figure 1.26***), a water soluble vitamin, is a widely used labelling group in nucleic acids. It is a convenient, versatile and easily handled molecule and its tight binding ($K_d = 10^{-15}M$) to the egg-white glycoprotein avidin or the non-glycosylated, bacterially derived analogue, streptavidin, is easily detected in highly sensitive enzymatic systems.

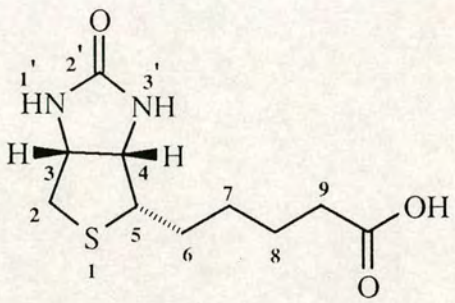


Figure 1.26 : (+)Biotin

The methods for biotinylating oligonucleotides are again numerous but to be of maximum value they have to be compatible with standard solid phase oligonucleotide synthesis. There are two principle strategies: the reaction of an amino functionalised oligonucleotide with the biotin as its activated N-hydroxysuccinimidyl ester derivative or the direct coupling of a biotin phosphoramidite monomer onto a support bound oligonucleotide.

One of the most common reasons for introducing a terminal amino group into an oligonucleotide is for reaction with a biotin derivative (see Section **1.4.4**, p36).[146,151-154] Amino groups can be added to individual nucleotides by means of base modifications to permit non-terminal, site specific introduction of one or more biotin groups to oligonucleotides.[155,156]

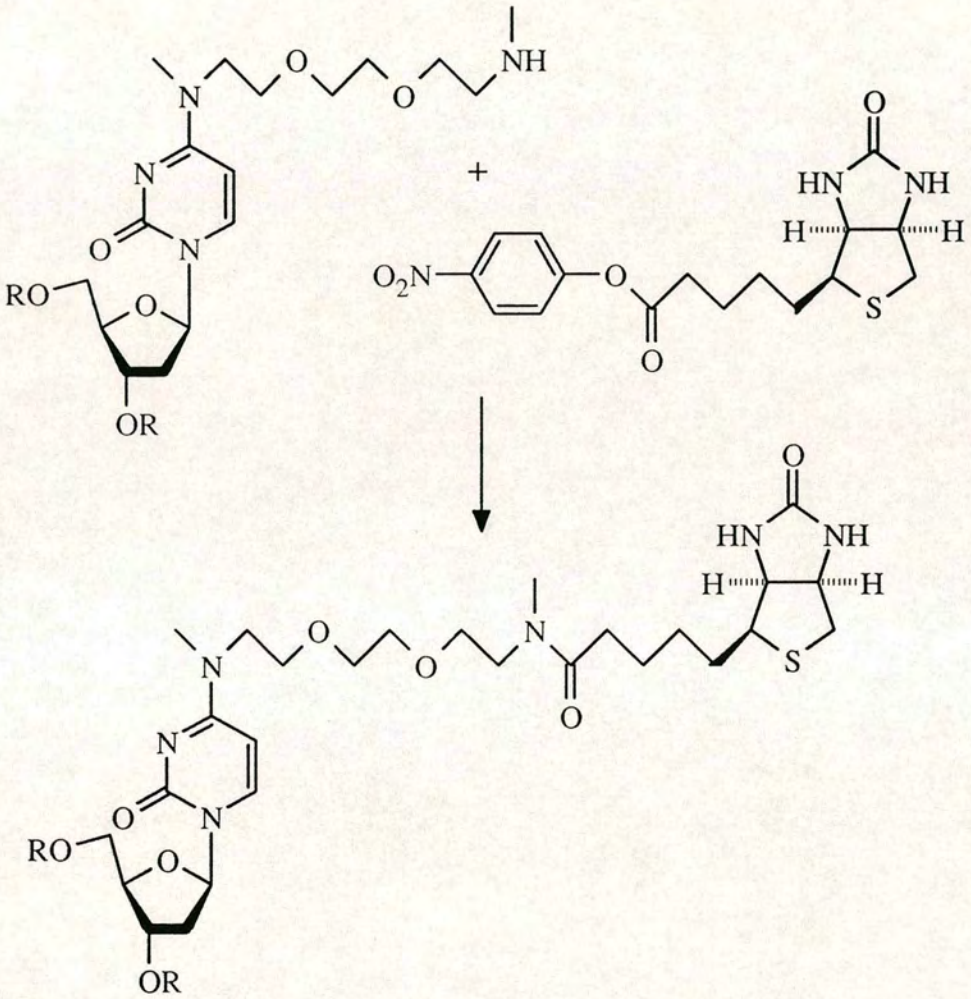
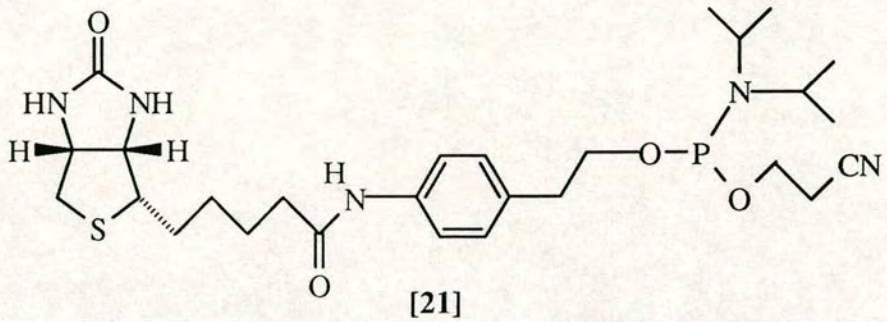
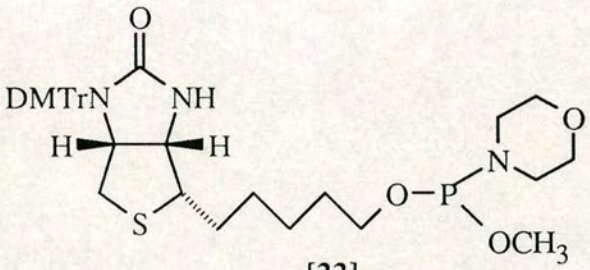


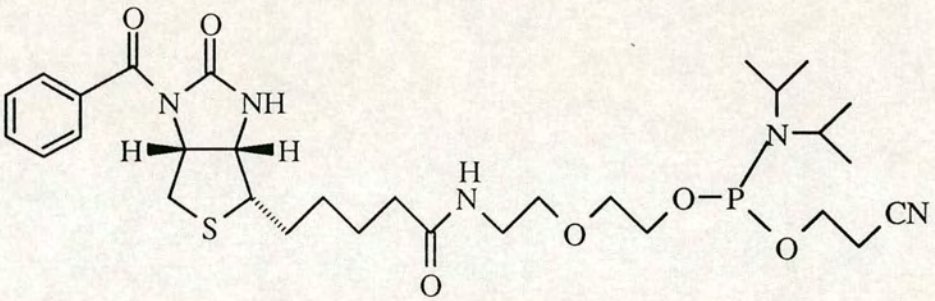
Figure 1.27 : Biotin Modified Nucleotide [157]



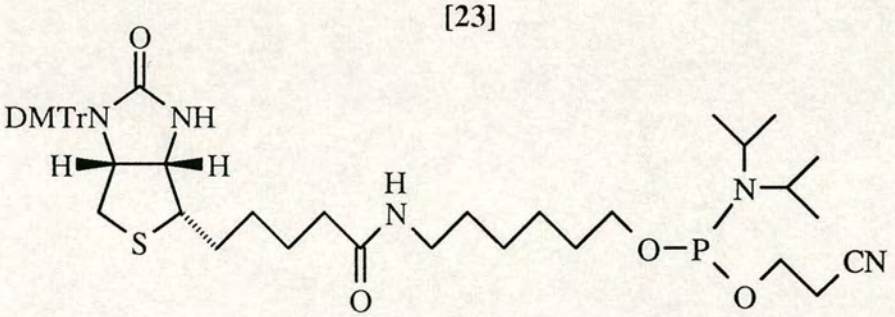
[21]



[22]



[23]



[24]

Figure 1.28 : Biotin Phosphoramidite Monomers [158-161]

For example, the introduction of a 1,2-bis(2-methylaminoethoxy)ethane to the 4 position of thymidine and the addition of the *p*-nitrophenyl biotin active ester (***Figure 1.27***).[157] The modification of a sugar ring has also been used to introduce an amino group via the periodate oxidation of a 5'-terminal uridine residue.

Biotin phosphoramidites (***Figure 1.28***) are far more useful since no aminolink synthesis is necessary, no awkward base modification processes are required and direct incorporation during oligonucleotide assembly occurs without any post-synthetic procedures. Furthermore, if the 1' nitrogen of the biotin ring is protected with a DMTr group, coupling efficiencies can be measured. Cocuzza[158] introduced a *p*-aminophenethyl group to the biotin active ester and then converted this to a phosphoramidite **[21]**. Alves *et al*[159] reduced the side chain carboxylic acid on biotin to the corresponding alcohol via the methyl ester and then phosphitylated it to give **[22]**. However, the monomers were insoluble in acetonitrile, the universal solvent of DNA synthesis and quoted coupling yields were low.[159] Will[160] synthesised two biotin monomers containing 3-oxapentyl **[23]** and octamethylene spaced side arms. These were acetonitrile soluble and coupled with extremely high yields. However, the most widely used monomer, again acetonitrile soluble and offering high coupling efficiencies, was synthesised by Pon[161] by the addition to biotin of *t*-butyldimethylsilyl protected aminohexanol followed by phosphitylation of the deprotected hydroxyl group **[24]**.

An important application of biotin is as a label for primers in the polymerase chain reaction (PCR), a DNA amplification technique. The PCR products are isolated on streptavidin, where binding of the biotinylated DNA is so strong that recovery of the DNA can pose a problem. A cleavable biotin functionality[132] offers a solution to this problem.

2 RESULTS AND DISCUSSION

2.1 Oligonucleotide Cellular Uptake Enhancement

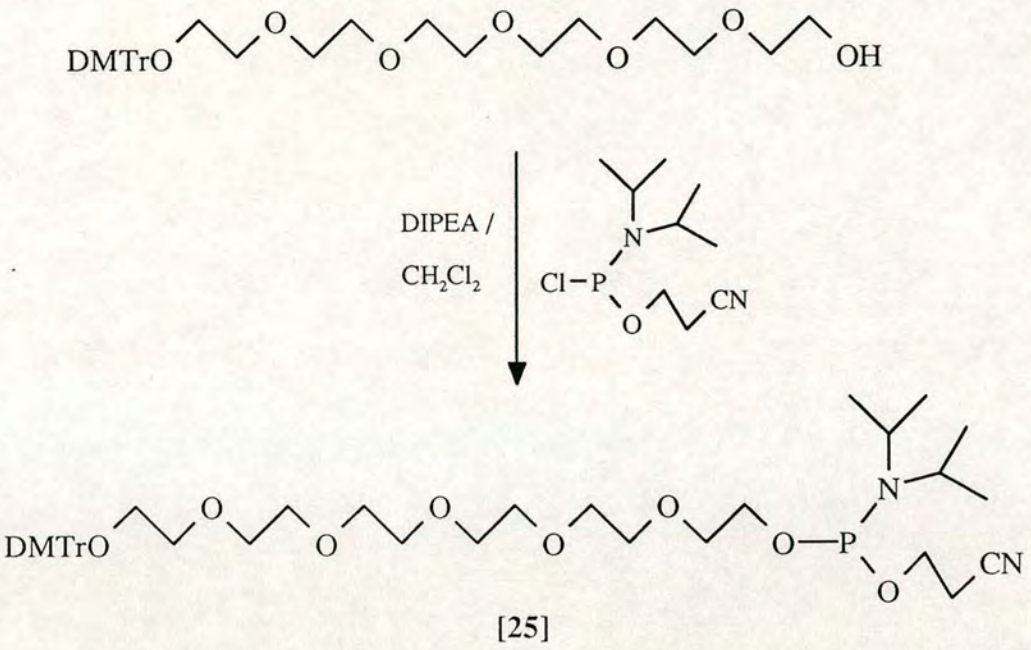
The 28mer phosphorothioate, SRev, has been shown to have potent antiviral activity against the human immunodeficiency virus (HIV).[61] However, this activity is limited by the poor cell permeability properties of the oligonucleotide. It has already been shown that the attachment of a lipophilic group such as cholesterol improves the antiviral activity of SRev.[106-108] This increased activity is the result of the interaction of the lipophilic cholesterol group with the cell membrane leading to cell encapsulation (endocytosis) of the oligonucleotide.

A drawback to the above approach is that once within the cell, the oligonucleotide is still linked to the cell membrane via the lipophilic group, inhibiting it from hybridising with the target mRNA. Therefore, in an attempt to address this problem, a monomer containing a disulphide group was synthesised. As it has already been shown that a disulphide group is reduced by cellular enzymes but is stable outside the cell,[112] the disulphide containing monomer was introduced between a lipophilic group and the oligonucleotide. The disulphide group was to act as a cleavable linker, releasing the oligonucleotide from the transport agent once cell penetration had occurred. It was hoped that the anti-HIV activity of SRev would be significantly increased.

2.1.1 Synthesis and Coupling of Transport Agents

Initially it was decided to study those lipophilic groups which had already been employed in previous antisense cellular uptake studies.

Aminohexyl spaced cholesterol **[3]** (p24, Chol-C6) and 4,4'-dimethoxytrityl (DMTr) protected hexadecyl **[5]** (p26, C16) phosphoramidite monomers were synthesised according to the method of MacKellar *et al.*[104] To act as a lipophobic control, DMTr protected hexaethyleneglycol phosphoramidite monomer **[25]**, (HEG) was prepared (***Figure 2.1***).[162]



[25]

Figure 2.1 : Hexaethyleneglycol Monomer

The 1,2-di-O-hexadecylglyceryl molecule **[4]** was synthesised according to a literature method.[115-119] However, when the subsequent phosphoramidite monomer (C35) was coupled onto the 5'-terminus of a standard oligonucleotide, problems identical to those previously encountered with cholesterol[104] were observed. The steric bulk of the 1,2-di-O-hexadecylglyceryl group inhibited the iodine oxidation step of the coupling cycle so that extended treatment was required to achieve quantitative reaction. More seriously, steric hindrance completely inhibited the action of tetraethylthiuram disulphide (TETD) when a sulphurisation step was carried out.

As a result, an aminohexyl spaced molecule was synthesised (***Figure 2.2***). Compound **[4]** was reacted with bis(*p*-nitrophenyl)carbonate **[26]** in dichloromethane in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to give the carbonate **[27]**. Subsequent reaction with 6-aminohexan-1-ol in dichloromethane in the presence of triethylamine (TEA) gave the aminohexyl spaced product **[28]** which was then phosphitylated to give the phosphoramidite monomer **[29]**, (C35-C6).

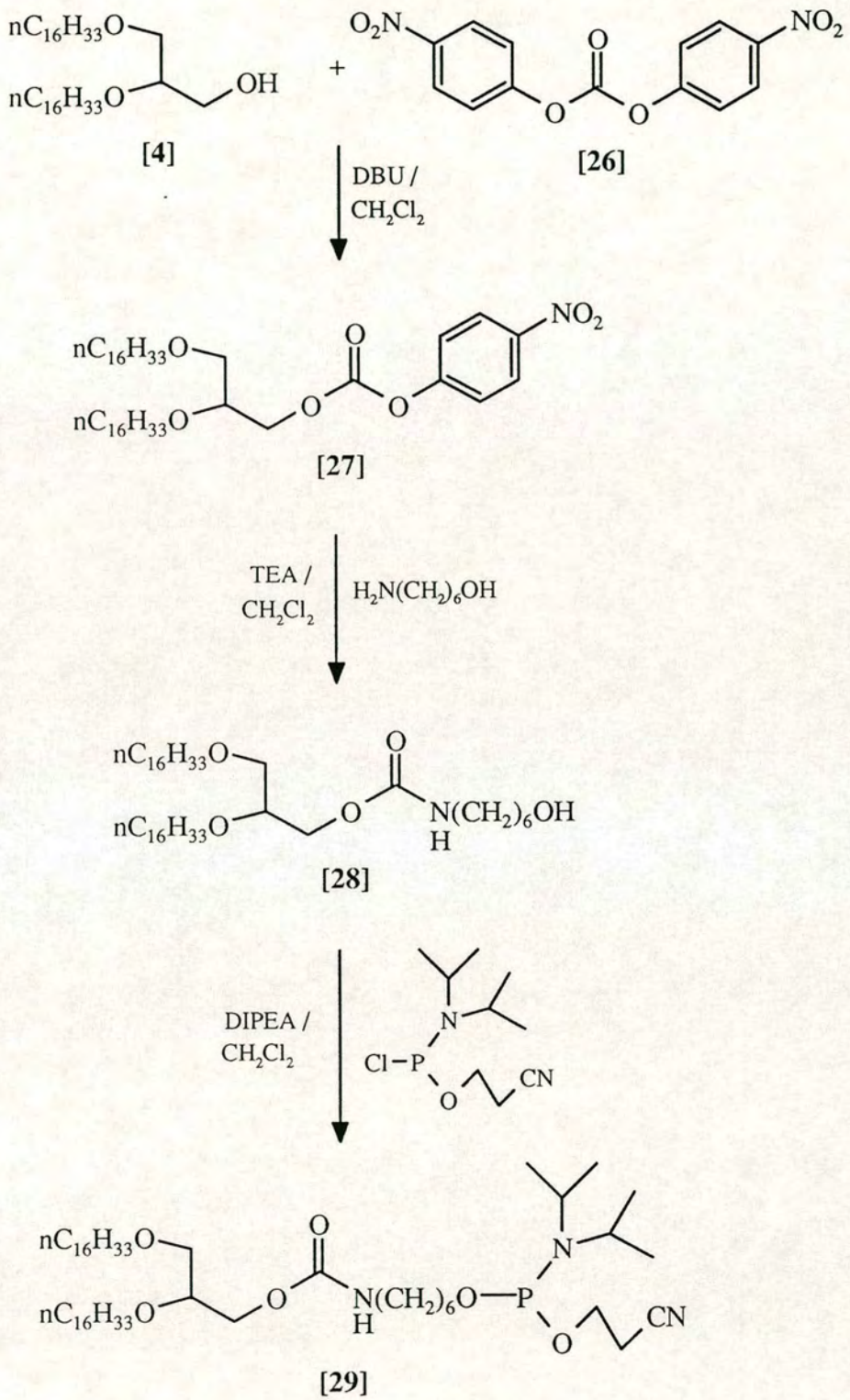


Figure 2.2 : Aminohexyl Spaced 1,2-di-O-hexadecylglyceryl Monomer

Phosphitylation was carried out in anhydrous dichloromethane in the presence of diisopropylethylamine (DIPEA). The phosphitylating reagent, cyanoethyl-N,N-diisopropylchlorophosphoramidite, was prepared by the standard procedure.[163] Prior to flash chromatography purification of the product **[29]**, the silica-gel was washed with anhydrous triethylamine (TEA) to neutralise any acidic groups which might protonate the phosphoramidite monomer and lead to decomposition of the product. This procedure was adopted for all flash chromatography purifications involving a phosphoramidite or a functionality protected by the acid labile DMTr group.

In all cases, purity of the phosphoramidite monomers was confirmed by phosphorus NMR (e.g. compound [29], *Figure 2.3*).

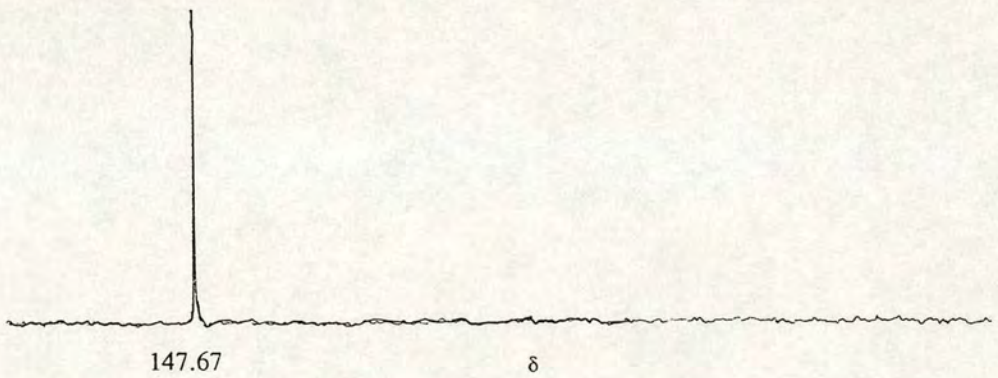


Figure 2.3 : ^{31}P-NMR of [29]

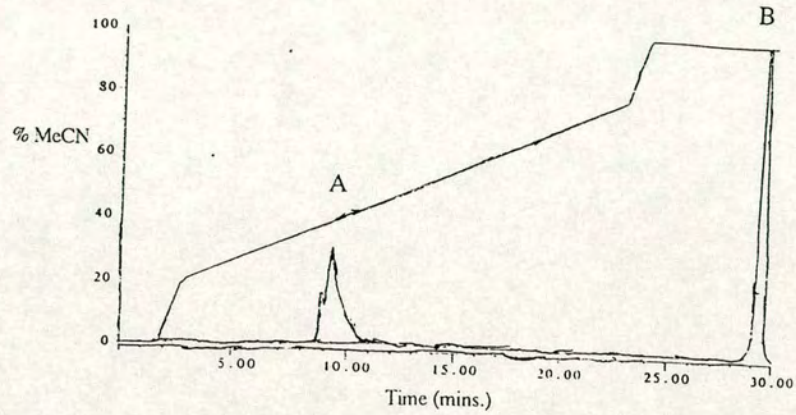
The synthesis of SRev and other phosphorothioates is an expensive and time consuming procedure because of the repeated sulphurisation steps required. Initially, therefore, it was decided to attempt automated, solid phase coupling of the monomers on the 5'-termini of short oligo dT sequences using standard phosphoramidite chemistry. Owing to lack of solubility in acetonitrile, all the hydrophobic monomers were dissolved in anhydrous dichloromethane prior to coupling. Extra

dichloromethane column washes were used in the synthesis cycle to prevent precipitation of the monomers and blockage of the reagent lines. In addition, extended coupling cycles were used; in particular an extension of the oxidation step to avert the problems that might be caused by steric hindrance (see Section **3.2**, p115).

Table 2.1

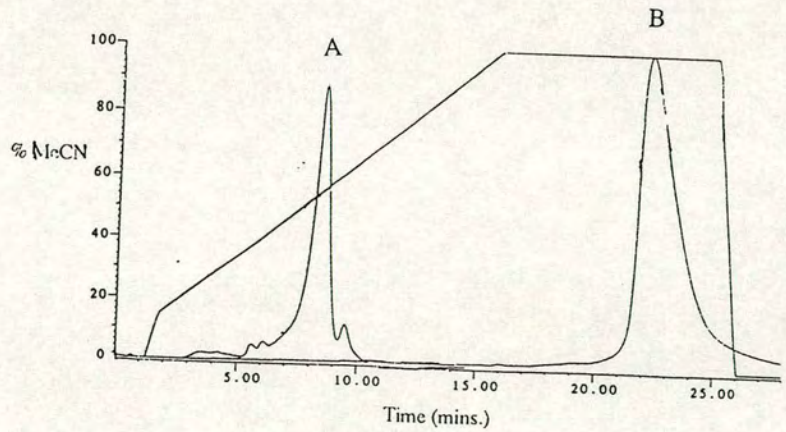
Phosphoramidite	**3**	**5**	**25**	**29**
Coupling Efficiency (%)	93	94	79	85
Terminal dT Coupling (%)	N/A	93	89	N/A

The coupling efficiencies obtained (***Table 2.1***) were all confirmed by reverse phase HPLC analysis (e.g. compound **[29]**, ***Figure 2.4***, using Gradient **1**, p120) with the product (peak B) eluting considerably later than the failure sequences (A). Where the monomer was hexadecyl **[5]** or hexaethyleneglycol **[25]**, detritylation led to the exposure of a further 5'-hydroxyl functionality. To confirm the success of the monomer couplings, a standard 2'-deoxythymidine phosphoramidite monomer was coupled onto the 5'-termini of oligonucleotides bearing detritylated HEG or C16 groups. The coupling efficiencies thus obtained by coupling onto these groups are indicated in ***Table 2.1*** (for the methods used to calculate the coupling efficiencies, see Section **3.2**, p115).



A : Failure Sequences B : C35-C6-dT5

Figure 2.4 : C35-C6 + dT5



A : Failure Sequences B : C35-C6-SRev

Figure 2.5 : C35-C6 + SRev

The coupling efficiencies were considered sufficiently high for all the monomers to be subsequently used in the synthesis of SRev and analogues of SRev. The same oxidation cycle could be used for the coupling of the lipophilic monomers onto the phosphorothioate 28mers but the resulting phosphodiester linkages would be vulnerable to hydrolysis by DNase enzymes. Sulphurisation with TETD to give the enzymatically stable phosphorothioates was therefore carried out. This proceeded satisfactorily for the aminohexyl spaced 1,2-di-O-hexadecylglyceroxy monomer **[29]**, as confirmed by HPLC analysis (e.g. **[29]**, ***Figure 2.5***, Gradient **1**).

As an antisense control, the phosphoramidite monomers **[3]**, **[5]** and **[29]** were coupled to the 5'-terminus of two other 28mer phosphorothioates. The two sequences were SRandom, d(CTG GTC TCG TTC GCC TCT CTT CTC CCG A), containing the same base composition as Srev but with a scrambled sequence and an oligo dC 28mer phosphorothioate (SdC28). Both of which would be unable to hybridise to the target mRNA on the *rev* gene. Again, in each case the final sulphurisation step proceeded as standard with HPLC traces showing the yields of the desired oligonucleotides to be equivalent to the SRev examples.

2.1.2 Synthesis of Disulphide Phosphoramidites

In the majority of literature examples, the disulphide group is introduced into an oligonucleotide by the reaction of an amino functionalised oligonucleotide,[129-132] by chemistry involving a disulphide exchange reaction[113,124,125,130] or by solid support modification.[124-127] Relatively straightforward means exist to obtain a 3'-disulphide[126] but only H-phosphonate chemistry has ever been employed to introduce a disulphide monomer at the 5'-terminus.[112]

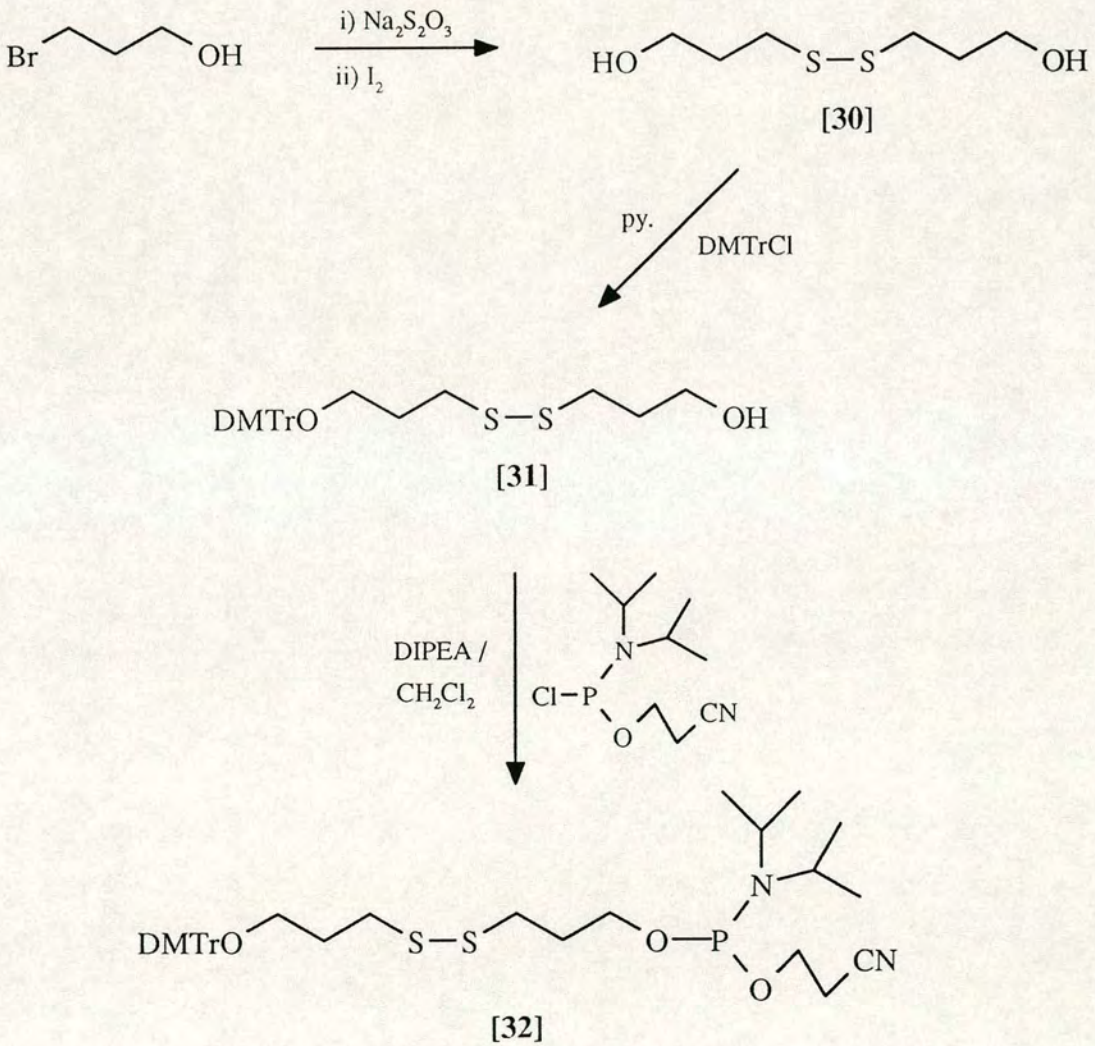


Figure 2.6 : Disulphide Phosphoramidite Monomer

A simple, disulphide containing phosphoramidite monomer would offer a superior methodology for 5'-disulphide incorporation into oligonucleotides.

A propyl linked disulphide unit **[30]** (***Figure 2.6***) was prepared from 3-bromopropan-1-ol using Bunte salt chemistry.[164,165] Sodium thiosulphate was used to convert the bromo alcohol to the thiol alcohol which was then oxidised to the disulphide by iodine in an ethanol/water solvent. Without further purification, this was reacted with an equivalent of 4,4'-dimethoxytrityl chloride in pyridine. Careful purification led to the product alcohol **[31]** which was then phosphitylated to the phosphoramidite monomer **[32]**. As is standard for phosphoramidites, purification of the monomer **[32]** was carried out rapidly to avoid oxidation. Fortunately, compound **[32]** possessed good solubility in acetonitrile and so standard coupling cycles could be used.

However, despite being a single spot on thin layer chromatography (tlc), the phosphorus-NMR spectrum of the purified monomer **[32]** indicated the presence of a large number of impurities. Solid phase coupling of the monomer as a 0.1M solution in acetonitrile onto the 5'-terminus of a dT sequence 5 nucleotides in length was low (52%) and the problem was assumed to be the result of disulphide cleavage by phosphorus (III) compounds present (***Figure 2.7***).

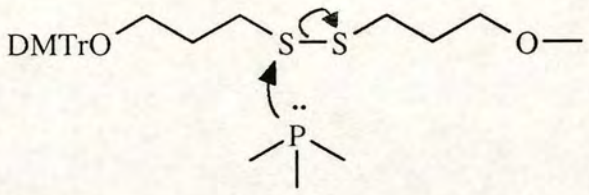


Figure 2.7

Throughout the phosphitylation procedure to prepare monomer **[32]**, the disulphide unit was exposed to disulphide cleavage conditions. Both the phosphitylating reagent and the monomer itself contain a phosphorus atom in its trivalent state capable of reducing the disulphide bond.

In an attempt to limit the exposure of the disulphide unit to the phosphitylating reagent, reaction times for the phosphitylation step were substantially reduced from the standard 1 hour down to 20 minutes. Again, on tlc, the product appeared as a single compound but on this occasion, the coupling efficiency was much improved (82%). As before, however, there was evidence for P(III) catalysed disulphide reduction. After being left for 30 minutes on the DNA synthesiser in solution, the disulphide monomer was coupled again onto an identical oligo dT sequence to give a coupling efficiency of 51%. Clearly, as compound **[32]** is a phosphoramidite monomer, it contains a P(III) atom and this atom is capable of reducing the disulphide unit. Whenever an attempt is made to use the monomer, this self-cleaving process is unavoidable and poses a considerable problem.

Nevertheless, further attempts were made to synthesise a purer sample of **[32]**. ^{31}P-NMR spectra always showed the presence of a characteristic phosphoramidite peak (δ 147-150), but also present were numerous other unidentified signals which did not correlate to any obvious side-product. However, coupling efficiencies were low and remained low (30-50%) despite attempted modifications to the monomer synthesis or the DNA synthesis cycle: for example, reducing the phosphitylating reaction times still further, extending the automated coupling cycle for the monomer and even changing to the less reactive phosphitylating reagent, 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphoramidite.

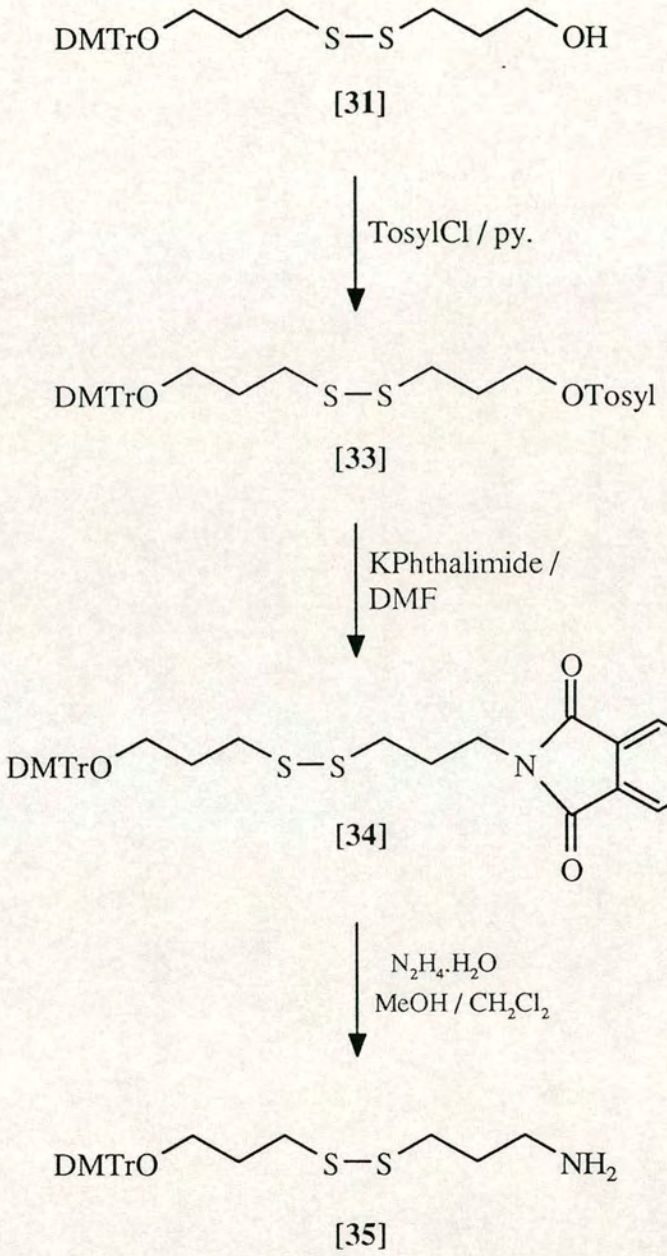
A further problem with the disulphide monomer **[32]** is that once it has been coupled to the support bound oligonucleotide it is necessary to couple onto it a lipophilic phosphoramidite monomer, thereby exposing the disulphide unit to further P(III) containing compounds. It was expected that any further coupling would also

be of substantially reduced efficiency and although significantly less pronounced than predicted, this was later shown to be the case.

As a means of circumventing this problem, it was decided to combine the disulphide and lipophilic groups in the same monomer. Hence only one coupling reaction would be required instead of two. Initially, the cholesteryl and 1,2-di-O-hexadecylglyceroxy groups were selected to test this idea. A similar strategy was adopted to the syntheses of the aminohexyl linked monomers **[3]** (p24) and **[29]** (p45).

The partially protected disulphide unit **[31]** was converted into its analogous amine **[35]** using the Gabriel synthesis and following a procedure based on that of Will (***Figure 2.8***).[160] The terminal hydroxyl group was reacted with tosyl chloride in pyridine to give the tosyl derivative **[33]**. The tosyl group was then displaced by potassium phthalimide in N,N-dimethylformamide (DMF) to give the phthalimide **[34]**. Reduction with hydrazine hydrate was carried out in a (1:1) dichloromethane/methanol mixture (owing to the lack of solubility of **[34]** in methanol alone).

The resulting amine **[35]** was then reacted with 1,2-di-O-hexadecylglyceroxy(*p*-nitrophenylcarbonate) **[27]** (***Figure 2.9***) and cholesteryl chloroformate **[1]** (***Figure 2.10***) respectively to give the products **[36]** and **[38]**. Detritylation was carried out with 5% trichloroacetic acid (TCA) in dichloromethane. The cholesteryl derivative **[38]** deprotected smoothly to give the product **[39]**. The 1,2-di-O-hexadecylglyceryl derivative **[36]**, however, gave two products under deprotection conditions. One of these was shown to be the thiol resulting from cleavage of the disulphide bond, although the reasons for this side reaction remain unclear. Interestingly, absolutely no analogous product was observed when the cholesteryl derivative was treated with TCA. The yield of the desired product **[37]** was extremely low(38%).


DMTrO
S—S
OH
[31]
TosylCl / py.
DMTrO
S—S
OTosyl
[33]
KPhthalimide /
DMF
DMTrO
S—S
N
O
O
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$N_2H_4.H_2O$
MeOH / CH_2Cl_2
DMTrO
S—S
NH_2
[35]

Figure 2.8

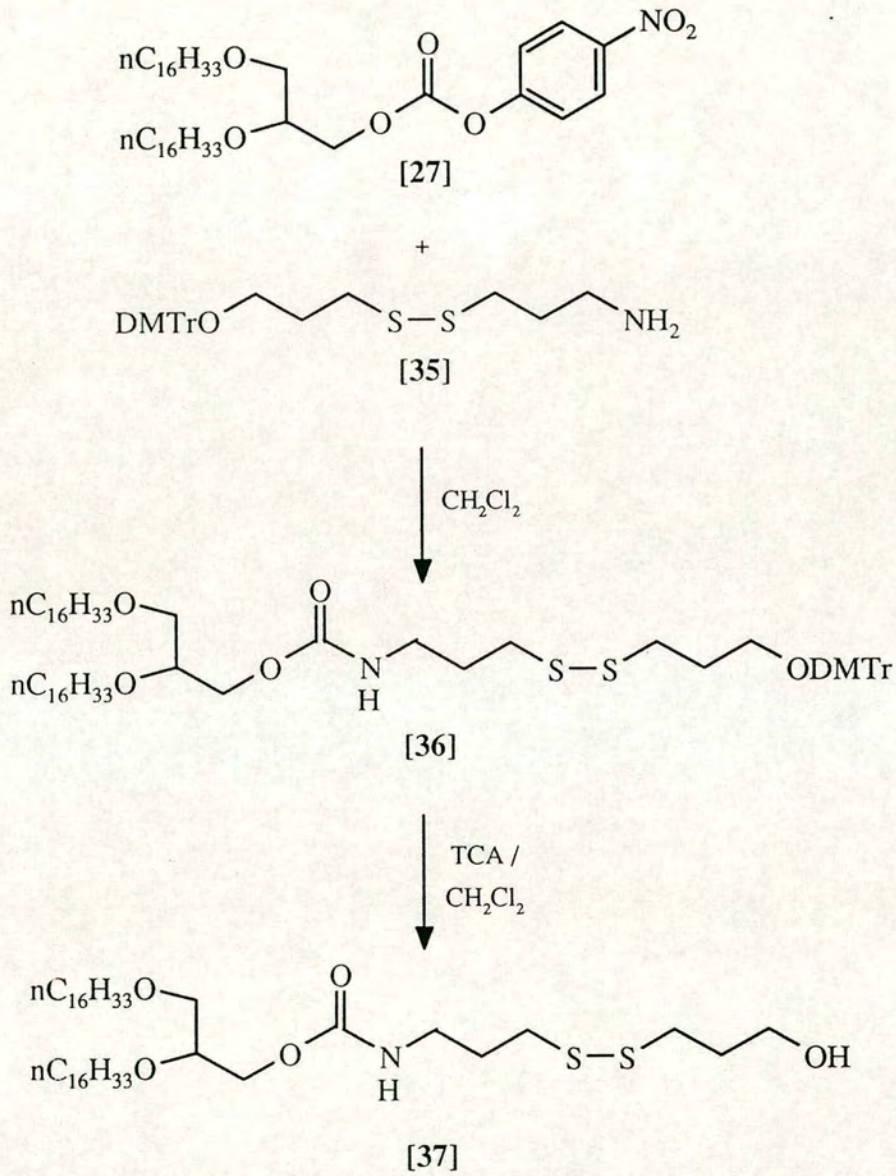


Figure 2.9 : 1,2-Di-O-hexadecylglyceryl Disulphide

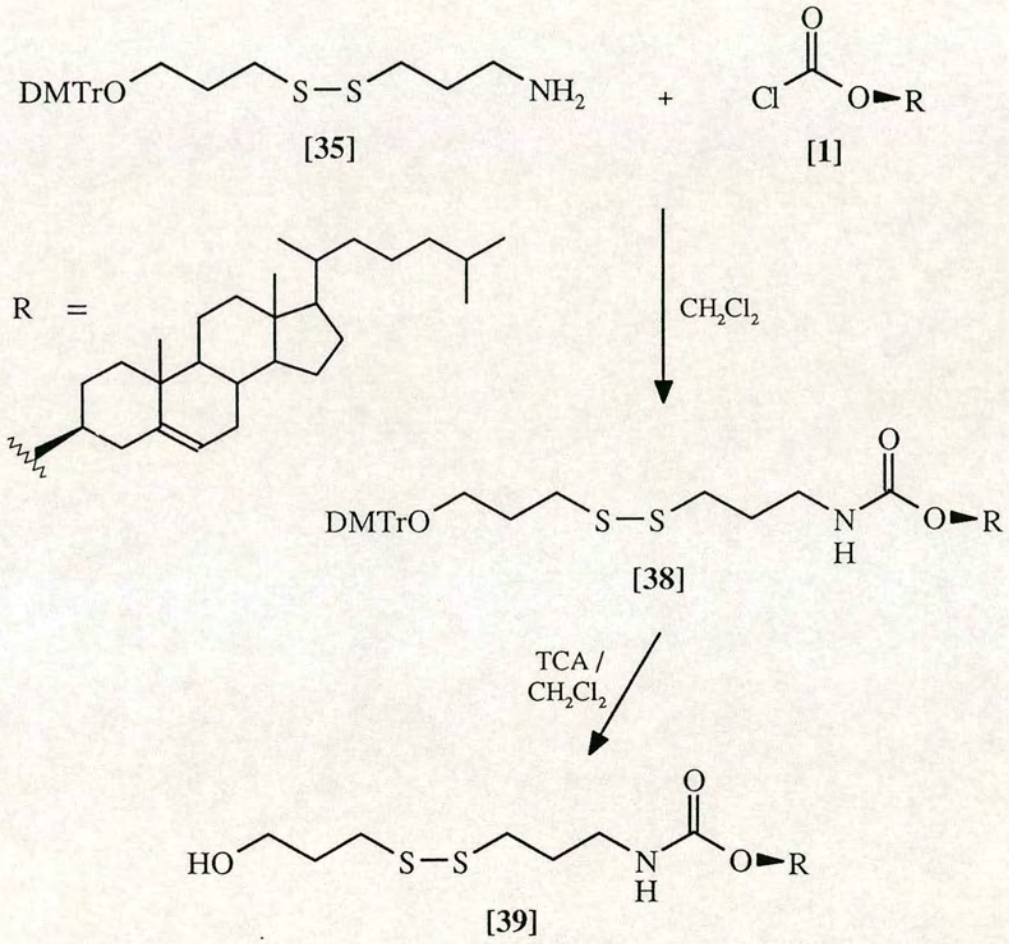


Figure 2.10 : Cholesteryl Disulphide

Further problems were encountered when the two compounds **[37]** and **[39]** were subjected to phosphitylation. In both cases, during the standard phosphitylation procedure, multiple products appeared on tlc. Even though the phosphoramidite targets appeared to be the predominant products, following purification, the ^{31}P-NMR spectra of both samples were complex, containing numerous signals, none of which could be assigned, but more significantly, there were no peaks at all in the phosphoramidite region. Attempted automated solid phase couplings onto dT 5mers in anhydrous dichloromethane indicated negligable coupling and no products were observed during subsequent HPLC analyses. Reduced reaction times during phosphitylation gave the same results.

Again, the reasons for these observations were unclear. Instead of sterically protecting the disulphide group during phosphitylation, the bulky cholesteryl and 1,2-di-O-hexadecylglyceryl groups appeared to destabilise the molecules. Clearly, they could not be used to synthesise the desired oligonucleotides.

2.1.3 The Phosphotriester Approach

2.1.3.1 Synthesis of Disulphide Phosphotriesters

It seems most likely that the lack of stability of a disulphide group in a phosphoramidite molecule is caused by disulphide reduction by P(III) species. During peptide synthesis, the thiol group of cysteine residues is often protected by converting it into a disulphide;[166] deprotection is achieved by reduction, usually with standard reducing agents such as sodium borohydride, but it can be achieved by using trialkyl phosphines e.g. n-tributylphosphine (TBP). It is therefore likely that any oligonucleotide synthesis approach involving disulphide groups should avoid the inclusion of any phosphorus atoms in the trivalent oxidation state. For that reason, it was decided to use phosphotriester chemistry. In this approach, all phosphorus atoms exist in their P(V) state throughout oligonucleotide assembly.

The same basic approach i.e. the synthesis of a simple monomer containing a disulphide group, was adopted as before. As in the phosphoramidite approach, phosphotriester chain extension is the 3' to 5' direction; therefore the coupling of such a monomer would again automatically be onto the 5'-terminus of an oligonucleotide. The basic disulphide unit **[31]** was subjected to a standard phosphotriester phosphorylation (***Figure 2.11***), a one-pot procedure in tetrahydrofuran (THF) in the presence of base (TEA). Once the product **[40]** had formed from the reaction between the disulphide compound **[31]** and the phosphorylating reagent, 2-chlorophenyldichlorophosphate, the reaction mixture was quenched with aqueous 1.0M triethylammonium bicarbonate (TEAB). With the phosphorylating reagent containing two chlorine atoms, both capable of acting as leaving groups, there was evidence (on tlc) for the disulphide unit **[31]** substituting at both positions. Exposure to aqueous TEAB ensured that this disubstitution occurred to only a minor extent.

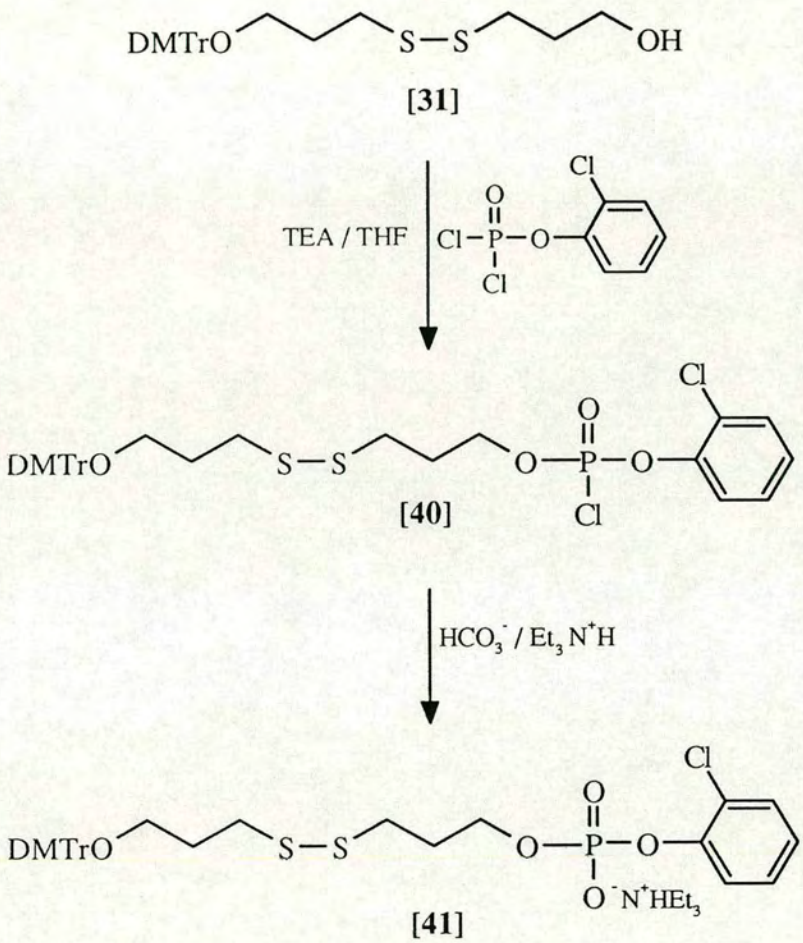


Figure 2.11 : Disulphide Phosphotriester Monomer

The phosphotriester monomer **[41]** thus synthesised was purified by silica-gel flash chromatography, on this occasion with the added precaution of having TEA present in the eluent throughout to ensure that the monomer remained as a triethylammonium salt. Attempts to convert the yellow oil to a solid foam were unsuccessful. Nevertheless, the characteristic phosphotriester peak (δ -4.49) observed on the ^{31}P-NMR (***Figure 2.12***) indicated that the monomer was of high purity.

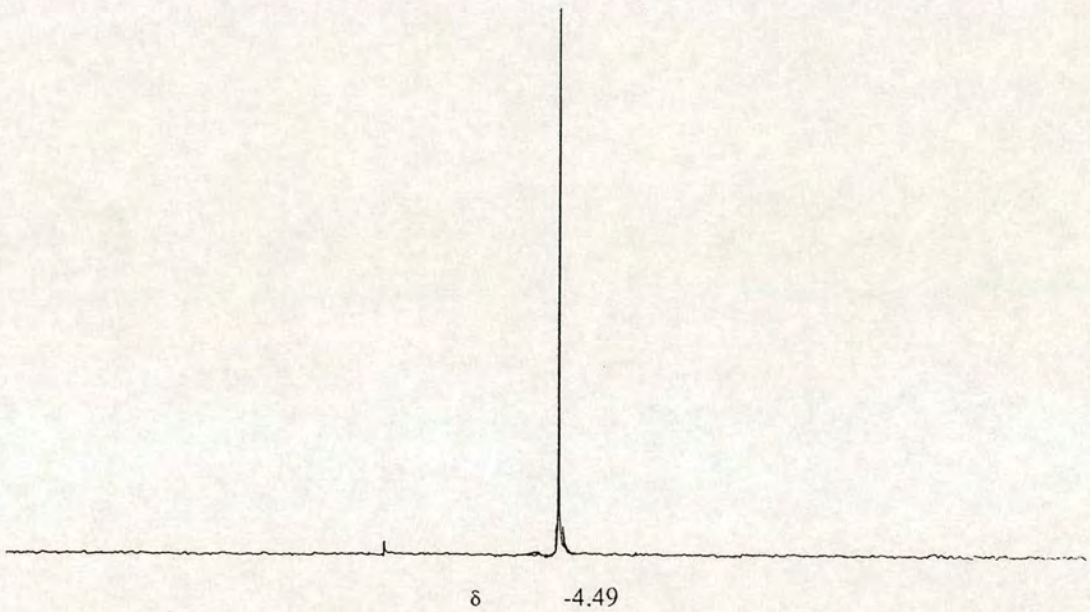


Figure 2.12 : ^{31}P-NMR of [41]

Modifying an automated DNA synthesiser to be compatible with phosphotriester chemistry is clearly perfectly feasible but it was decided to retain phosphoramidite chemistry for coupling of standard monomers. All phosphotriester monomers were added in a non-automated procedure. Coupling efficiencies of the triester monomer, outlined below (***Table 2.2***), were all obtained by returning the synthesis column to the DNA synthesiser once the phosphotriester monomer had been coupled and cleaving the terminal 4,4'-dimethoxytrityl group.

Table 2.2

Monomer (mg)	54	54	81	108	108	108	108
MSNT (eq.)	2	2	2	2	3	1	3
pyridine (%)	50	10	10	10	10	10	10
MeCN (%)	50	90	90	90	90	90	90
Coupling Time (mins.)	90	90	90	90	90	90	45
Coupling Efficiency (%)	69	75	88	92	95	31	68

Pyridine – doubly distilled prior to use – is the standard solvent in phosphotriester couplings but it tends to cause clumping of the CPG resin and blocking of the reactive sites. To avoid this, anhydrous acetonitrile (50%) was employed in the solvent mixture for the phosphotriester coupling procedure. The concentration of the monomer **[41]** was initially based on standard phosphotriester couplings (~0.1M)[167] and 2 equivalents of the condensing agent 1-(2-mesitylenesulphonyl)-1,2,4-triazole (MSNT) were used. A sufficient volume of solvent was taken (0.20ml) to fill the synthesis column and to ensure that all the air bubbles could be ejected. To ensure complete reaction, coupling times of 90 minutes were used.

With a coupling efficiency of 69%, the very first attempt to couple the monomer onto a dT 5mer sequence was extremely promising. However, extensive clumping of the solid support beads was observed. Continual agitation of the synthesis column was required to promote the reaction. The anhydrous acetonitrile content of the solvent mixture was increased still further (90%) and coupling of the monomer **[41]** was repeated. On this occasion, movement of the CPG resin within the synthesis column was unrestricted due to the increased MeCN content and only occasional agitation was required to obtain a coupling efficiency of 75%.

Increases in the concentration of the monomer solution, by 50% then by 100% over the original values, resulted in significant increases in the coupling efficiencies

recorded (88%, 92%). Fixing the monomer concentrations at 108mg (0.14mmol) per addition, the MSNT content was increased to 3 equivalents (0.42mmol). The resulting coupling efficiency (95%) was consistent to within 2% over two couplings attempted subsequently under identical conditions and was very close to the optimum possible for phosphotriester chemistry. It was certainly sufficient for the intended application and considerably superior to the phosphoramidite disulphide monomer. In addition, the phosphotriester disulphide monomer was stable over a considerable period of time, with no significant reduction in coupling efficiency observed after storage for 6 months at -6^{o}C.

That such a large quantity of MSNT (123mg) was required for each phosphotriester addition was confirmed by the considerable reduction in coupling efficiency observed (31%) when only one equivalent was used. This perhaps implies that moisture was present in the solvents or the monomer and that the MSNT was acting as a drying agent. However, the pyridine was doubly distilled and the triester monomer was dried overnight in a vacuum desiccator immediately prior to use to give conditions as close to anhydrous as possible.

The only drawback to the coupling procedure adopted was the 90 minute coupling time, significantly longer than a standard phosphotriester coupling cycle (40 minutes without N-methylimidazole).[33] The lower coupling efficiency obtained (68%) when the coupling time was reduced by 50% suggested that 90 minutes were indeed justified to ensure optimum coupling yields. To enable this time to be reduced, one equivalent of the catalyst, N-methylimidazole (0.14mmol, 11μl), was added to the solvent mixture prior to the coupling reaction. After a 30 minute reaction, subsequent analysis indicated a complete absence of any product. The N-methylimidazole was carefully distilled to remove any moisture which might have been interfering with the reaction. but the result was the same. Even a 90 minute coupling reaction with N-methylimidazole present yielded no product whatsoever. The N-methylimidazole appeared to inhibit the phosphotriester addition process for this monomer.

2.1.3.2 Cleavage of the Aryl Protecting Group

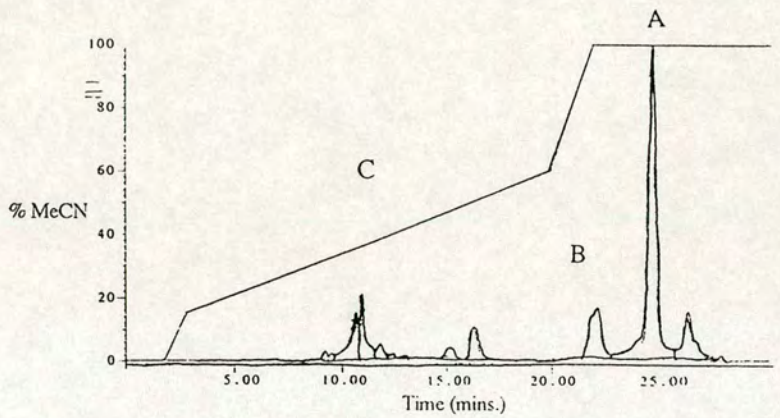
The *o*-chlorophenyl phosphate protecting group is usually removed by oximate anion[29] but cleavage can be carried out simply by heating with ammonium hydroxide.[30] In addition to being a simpler procedure, ammonium hydroxide treatment is compatible with the standard base deprotection protocol – heating at 55°C for 5 hours – which follows oligonucleotide cleavage from the solid support, therefore reducing the number of deprotection steps by one. It has been reported that ammonium hydroxide treatment causes some cleavage (2-3%) of the oligonucleotide backbone at every aryl protected phosphate.[30] However, when there is only one *o*-chlorophenyl group present in the oligonucleotide chain, as is the case here, this effect should be minimal.

To test this, an oligo dT sequence 15 nucleotides in length was synthesised and the disulphide phosphotriester monomer **[41]** (SS) was coupled to its 5'-terminus. On reverse phase HPLC, oligonucleotides elute according to their levels of hydrophobicity; if an oligonucleotide still carries a DMTr group, during HPLC analysis it elutes considerable later than its failure sequences enabling it to be more clearly visualised and more easily purified. Therefore, on this occasion, the DMTr protecting group was left on the oligonucleotide after synthesis.

Following automated cleavage from the solid support, the oligonucleotide was heated at 55°C in ammonium hydroxide and analysed on HPLC at one hourly intervals (***Figures 2.13-2.17***, Gradient **2**). As a consequence of using an oligo dT sequence, no base protecting groups were present and simplified HPLC traces were obtained. As can be clearly seen, the peak for the fully protected oligonucleotide (A) diminished as the product of *o*-chlorophenyl cleavage (B) increased with the failure sequences (C) showing little change. The reaction was almost complete after 3 hours and after 5 hours, negligible amounts of the *o*-chlorophenyl protected oligonucleotide remained. It was also clear that negligible cleavage of the oligonucleotide backbone by base occurred, as virtually no product loss was observed upon prolonged heating

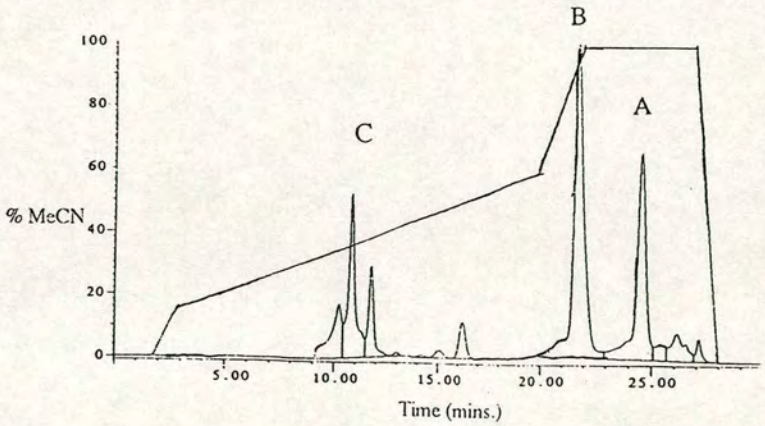
in aqueous ammonia. This stability was confirmed by showing that the HPLC trace of overnight heating in ammonium hydroxide was identical to that after heating for 5 hours.

Another sample of **[41]** coupled to a dT15 oligonucleotide was treated with 2-nitrobenzaldoxime in a dioxane/water mixture in the presence of base (1,1,3,3-tetramethylguanidine) at 37°C overnight[167] with the same result. Thus heating in ammonium hydroxide offered a simple, convenient means to cleave the aryl protecting group from the phosphotriester.



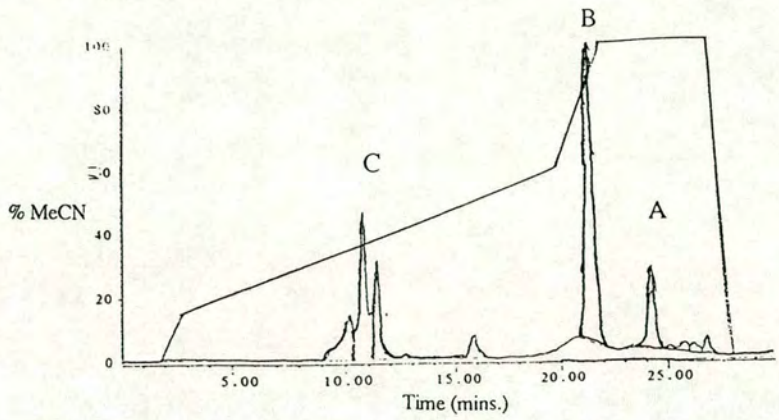
A : SS-dT15 + o-chlorophenyl B : SS-dT15 C : Failure Sequences

Figure 2.13 : SS + dT15



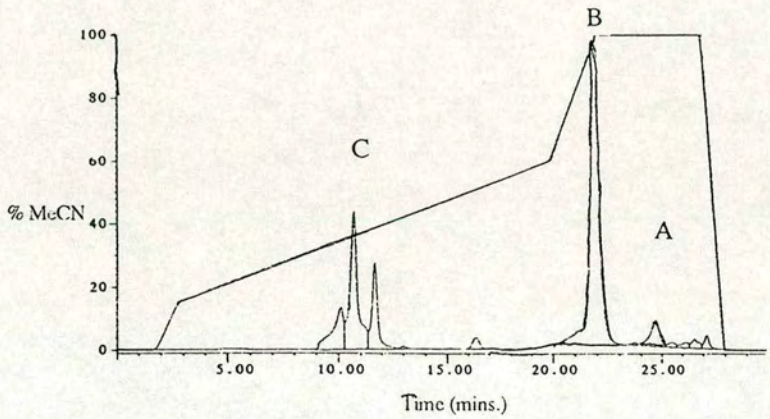
A : SS-dT15 + o-chlorophenyl B : SS-dT15 C : Failure Sequences

Figure 2.14 : SS + dT15 (1 hr., 55°C)



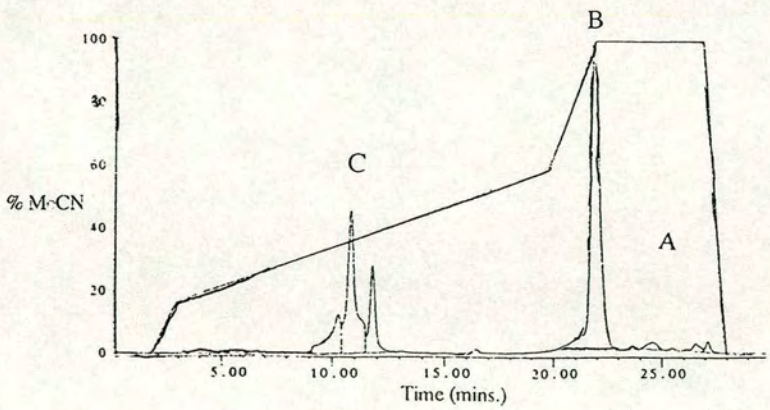
A : SS-dT15 + o-chlorophenyl B : SS-dT15 C : Failure Sequences

Figure 2.15 : SS + dT15 (2 hrs., 55°C)



A : SS-dT15 + o-chlorophenyl B : SS-dT15 C : Failure Sequences

Figure 2.16 : SS + dT15 (3 hrs., 55°C)

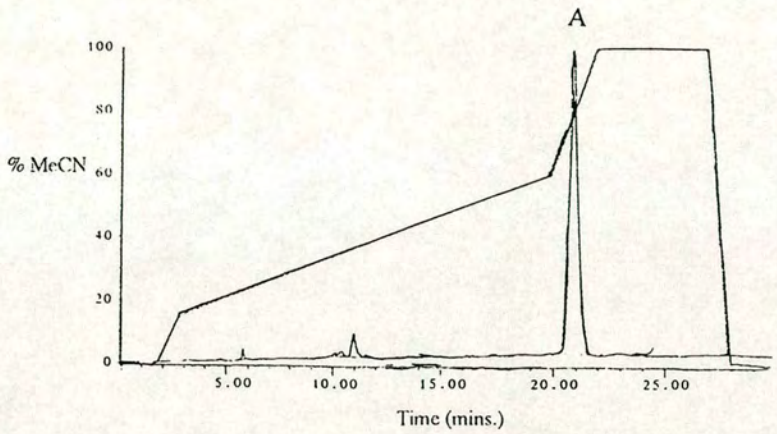


A : SS-dT15 + o-chlorophenyl B : SS-dT15 C : Failure Sequences

Figure 2.17 : SS + dT15 (5hrs., 55°C)

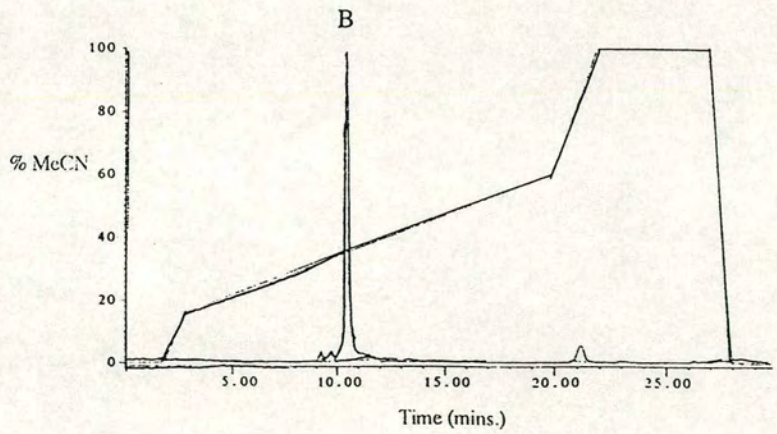
2.1.3.3 Cleavage of the Disulphide Bond

The standard method for disulphide bond cleavage involves treatment with dithiothreitol (DTT)[128] in the presence of base. The dT 15mer sequence with the disulphide phosphotriester monomer coupled to the 5'-terminus was made as before and the *o*-chlorophenyl group was removed with ammonium hydroxide. The product oligonucleotide was purified by reverse phase HPLC (***Figure 2.18***, peak A, Gradient **2**, p120) then treated with an excess of DTT (50mg, 0.32mmol) in aqueous buffer in the presence of ammonium hydroxide. After one hour, the sample was run down a Sephadex G25 gel filtration column. These nucleic acid purification (NAP) columns are used for oligonucleotides greater than 12 residues in length, retaining all small molecules and shorter oligonucleotides. In this way, excess DTT and the small DMTr protected propylthiol fragment produced by the cleavage reaction were removed. The resultant oligonucleotide, bearing a 5'-propylthiol group, was analysed on HPLC (***Figure 2.19***). The loss of the DMTr group from the 5'-terminus as a result of disulphide cleavage gave the product a far earlier elution time (B) than the starting material (A). Significantly, disulphide cleavage occurred practically quantitatively; virtually no trace of the starting material could be seen on the HPLC trace. When the product of cleavage was treated with dithio-bis-2-nitrobenzoic acid (Ellman's reagent), a bright yellow colour resulted, confirming the presence of a thiol group. Under the same conditions, the starting material (the uncleaved disulphide) gave no reaction.



A : SS-dT15

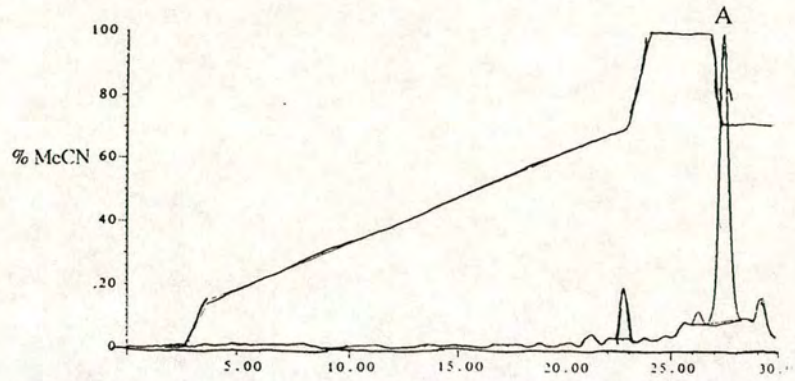
Figure 2.18 : SS + dT15



B : HS-dT15

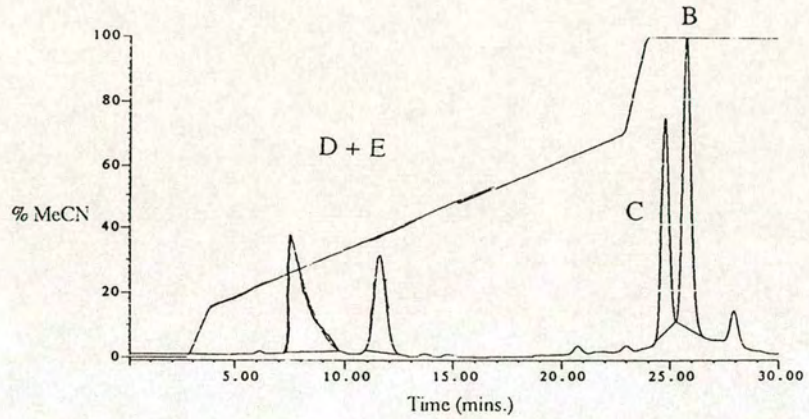
Figure 2.19 : (SS-dT15) + DTT

To further prove that disulphide bond cleavage was indeed occurring and simple detritylation was not responsible for the above observations, a disulphide group was incorporated within an oligonucleotide sequence. Again, an oligo dT sequence was synthesised, 10 nucleotides in length, and the phosphotriester monomer **[41]** was coupled onto the 5'-terminus using the standard procedure. On this occasion, however, upon returning the solid support to the DNA synthesiser, automated capping and detritylation were performed and a further six 2'-deoxythymidine residues were coupled on to give the sequence dT6-SS-dT10. Subsequent HPLC analysis of the deprotected product (peak A, ***Figure 2.20***, Gradient **3**, p120) indicated a high yield and when the product was subjected to DTT treatment, two clear products in virtually identical yield were observed (peaks B and C, ***Figure 2.21***). To enable both cleaved oligonucleotide strands to be detectable on HPLC, no gel filtration process was employed following DTT treatment since oligonucleotides shorter than 12 nucleotides in length are removed by gel filtration. For that reason, impurities arising from the presence of DTT were observed on the HPLC trace (peaks D and E). A mixed sample of the starting material and purified products was also analysed on HPLC and, although the concentrations of the samples differ, the 3 peaks (A, B and C) could quite clearly be seen (***Figure 2.22***); the oligonucleotide bearing a disulphide group and sixteen 2'-deoxythymidine residues eluted last (peak A), preceded by a dT 10mer bearing a 5'-propylthiol group (peak C) and a dT 6mer bearing a 3'-propylthiol group (peak B). Both of the cleaved fragments reacted with Ellman's reagent and the starting material gave no reaction. Furthermore, a dT16 sequence containing no disulphide units was synthesised; it showed no change following DTT treatment and did not react with Ellman's reagent.



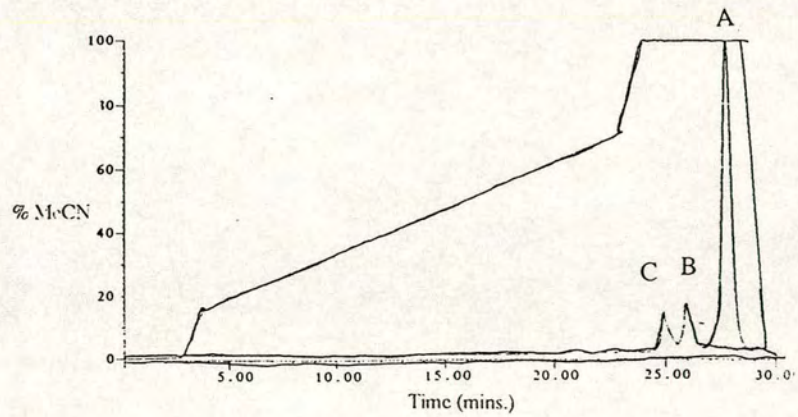
A : dT6-SS-dT10

Figure 2.20 : 5' dT6-SS-dT10 3'



B : HS-dT10 C : dT6-SH D + E : Excess DTT + Cleavage Products

Figure 2.21 : dT6-SS-dT10 + DTT



A : dT6-SS-dT10 B : HS-dT10 C : dT6-SH

Figure 2.22 : dT6-SS-dT10 + dT6-SH + HS-dT10

2.1.3.4 Oligonucleotides Containing 3'-Disulphides

Each of the six 2'-deoxythymidine residues coupled onto the disulphide unit during the course of the dT6-[**41**]-dT10 oligonucleotide synthesis above was monitored for coupling efficiency by trityl analysis. Each nucleotide was added as a phosphoramidite monomer and it was therefore expected that coupling efficiencies would be low. Interestingly, however, only the first nucleotide addition was poor (79% coupling efficiency) and every subsequent addition was of standard efficiency (98% average). A similar result was obtained – 83% for the first addition, an average of 97% for the rest – when the experiment was repeated. The addition of a standard phosphotriester monomer, in this case a 2'-deoxycytidine monomer, onto the disulphide under the same conditions used for the disulphide phosphotriester monomer, gave a coupling efficiency of 97% with all subsequent phosphoramidite additions again as standard (97% average).

A possible explanation for these observations is that after the first addition onto the disulphide, the disulphide group is subsequently sterically protected by the growing oligonucleotide chain from further P(III) attack. A more likely answer, however, is to do with the automated phosphoramidite coupling cycle. Every phosphoramidite monomer is treated with the activating agent, tetrazole, prior to exposure to the solid support bound oligonucleotide. In this way, due to protonation of the nitrogen atom of the diisopropyl group of the phosphoramidite moiety, the lone pair on the phosphorus atom is unavailable to participate in the disulphide bond reduction shown in ***Figure 2.7*** (p51). Possibly, a small proportion of the P(III) atoms of the phosphoramidite monomer are unprotonated by the tetrazole and therefore, at the time of exposure to the disulphide bond, the unprotonated monomer can still cause significant disulphide reduction. However, for all subsequent additions the disulphide bond is sufficiently sterically protected to inhibit this process. It is also possible that being primarily a polyalkyl chain, the disulphide unit, when at the 5'-terminus of an oligonucleotide, can adopt a variety of conformations, perhaps forming

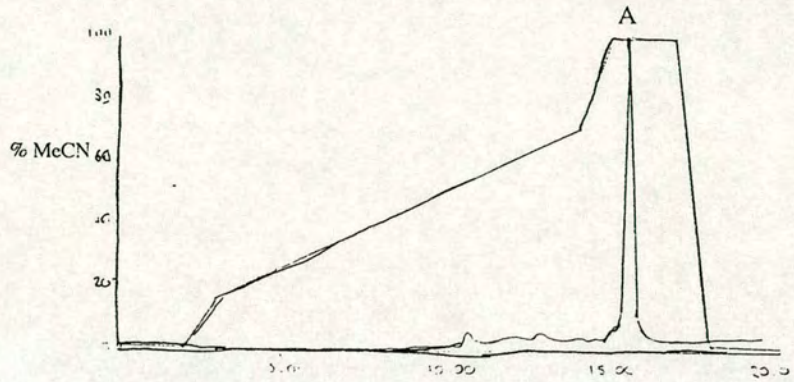
a hydrophobic pocket, hindering initial attack by P(III) but henceforth fixed into a rigid conformation. The results obtained when coupling onto the hexadecyl **[5]** and hexaethyleneglycol **[25]** groups with a 2'-deoxythymidine residue (see p47) seem to support this theory.

On a number of occasions,[124-127] a 3'-disulphide group has been introduced into an oligonucleotide by solid support modifications. In each case, no substandard coupling efficiencies were reported. It was decided to repeat this work by using the disulphide unit **[31]** (p50).

A standard controlled pore glass (CPG) derivatisation procedure was adopted. Aminopropyl functionalised CPG was washed with TCA then succinylated with succinic anhydride. The resultant carboxylic acid was activated with 4-dimethylaminopyridine (DMAP) and 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (DEC) hydrochloride and esterified by addition of the disulphide containing alcohol **[31]**. The unreacted succinate groups were then removed by treatment with piperidine and the unreacted amino groups were capped with acetic anhydride. A loading of 58μmol^{-1} of **[31]** on the CPG was obtained.

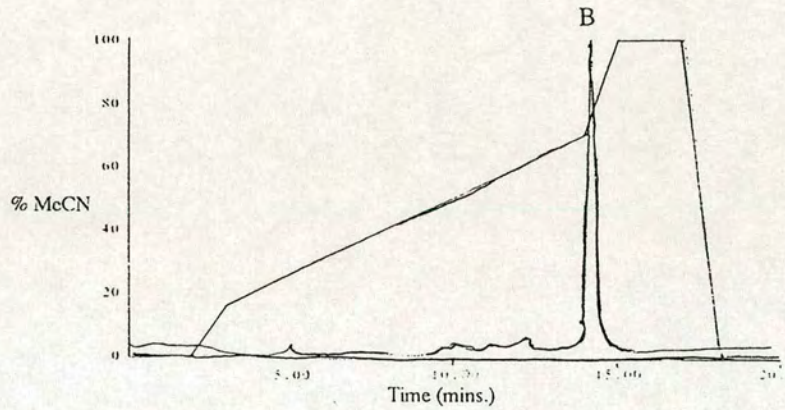
Approximately 8mg of the disulphide derivatised CPG resin were loaded onto a DNA synthesis column and a dT12 sequence was synthesised using standard phosphoramidite chemistry. Every single coupling efficiency was 98%±1%.

The product was analysed on HPLC (peak A, ***Figure 2.23***, Gradient **3**) then treated with DTT and gel filtered as before (peak B, ***Figure 2.24***). The difference in lipophilicity caused by having a disulphide group or a thiol at the 3'-terminus of an oligonucleotide is very small. However, the two oligonucleotides appeared to have slightly different elution times on HPLC under the same conditions (A, ~15.5mins. and B, ~14.5mins.) and although the analysis of a mixed sample shows only one main peak (A + B, ***Figure 2.25***), there was a definite shoulder on that peak, suggesting the presence of another, different oligonucleotide.



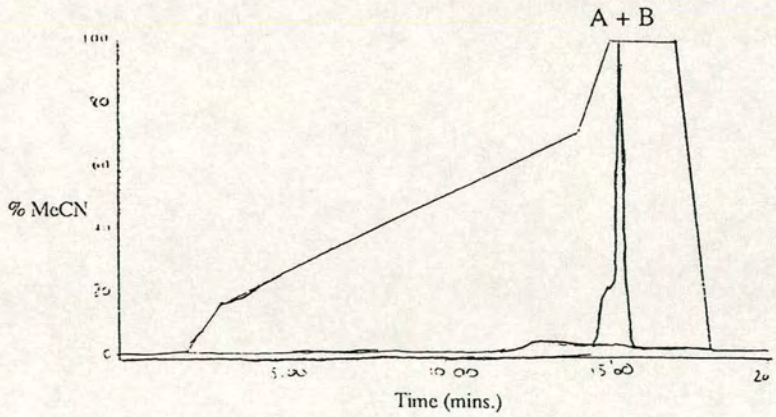
A : dT12-SS

Figure 2.23 : 5' dT12-SS 3'



B : dT12-SH

Figure 2.24 : (dT12-SS) + DTT



A + B : dT12-SS + dT12-SH

Figure 2.25 : dT12-SS + dT12-SH

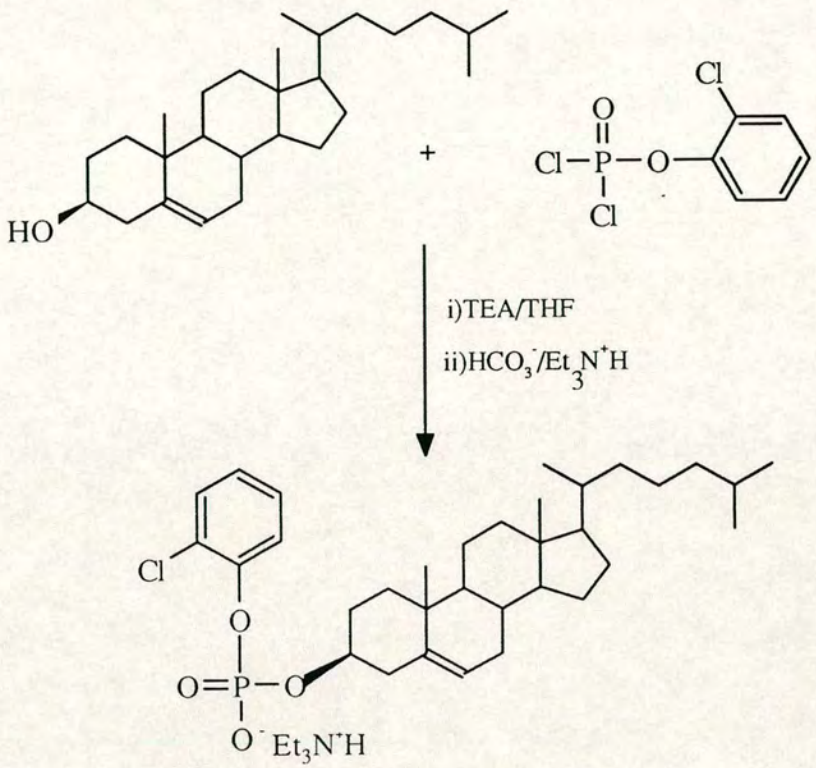
In addition, the product of the cleavage reaction reacted with Ellman's reagent whereas the starting oligonucleotide did not.

It can therefore be concluded that phosphoramidite couplings onto an oligonucleotide bearing a 3'-alkyl disulphide group pose no significant problems. Where the disulphide group is bound directly to the solid support and is therefore more hindered, even the first phosphoramidite addition onto the disulphide group proceeds well.

2.1.3.5 Cleavable Cholesterol Groups

Having established a consistent, high yielding method for the addition of a disulphide unit onto an oligonucleotide, a means was sought for the introduction of a lipophilic group. It had already been shown that the coupling of a phosphotriester monomer onto a 5'-terminal disulphide (dC, 97%) gives a superior yield to a phosphoramidite monomer coupling (dT, 79-83%). Thus it was decided to start by synthesising a cholesterol phosphotriester monomer.

The phosphorylation procedure (***Figure 2.26***) on this occasion required an overnight reaction due to steric inhibition of the reaction by the cholesterol moiety. However, for the same reason, no disubstitution by the cholesterol group on the phosphorylating reagent had occurred. The product **[42]** (Chol) was isolated as a white solid in a moderate yield (68%). The only change to the coupling procedure was the replacement of anhydrous acetonitrile by anhydrous THF in the solvent mixture to aid solubility. The same quantitiy of monomer (0.14mmol, 94mg) and MSNT (3 equivalents) were used as for the disulphide phosphotriester couplings. Initially coupling was attempted onto the 5'-hydroxyl group of an oligo dT sequence 15 nucleotides in length.



[42]

Figure 2.26 : Cholesterol Phosphotriester Monomer

Unfortunately, a coupling efficiency of only 31% was obtained. When gentle heating was applied to the synthesis column during the reaction (50°C for 30 minutes) the coupling efficiency was only marginally improved (46%). The problem was assumed to be the result of steric hindrance by the bulky cholesterol group. Even when the cholesterol phosphotriester monomer was coupled onto an oligonucleotide bearing a 5'-terminal hydroxy dipropyldisulphide group, less hindered than a terminal 2'-deoxythymidine residue, there was no real improvement in coupling efficiency (51%). Attempts to increase the duration of the coupling reaction or increasing the concentration of the monomer present both resulted in no improvement in coupling efficiency.

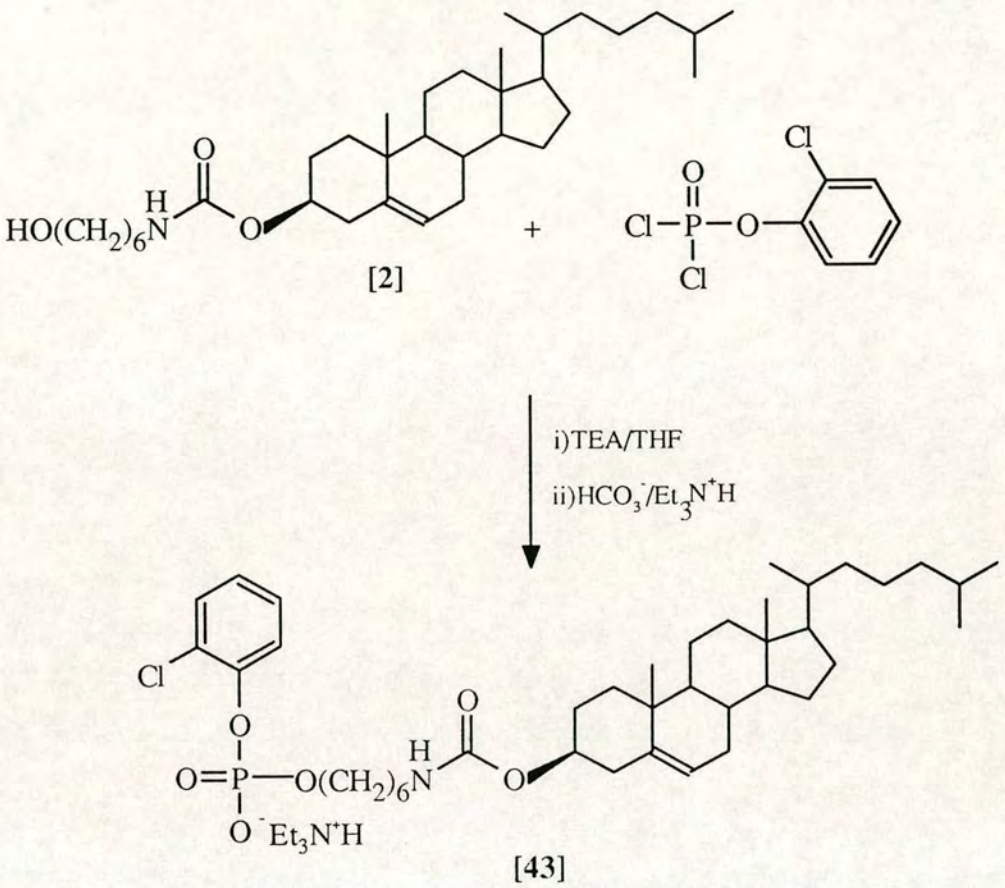


Figure 2.27 : Aminohexyl-Cholesteryl Phosphotriester Monomer

Next, the aminohexyl spaced cholesterol molecule **[2]**[104] was phosphorylated (***Figure 2.27***). Only a slightly extended phosphorylation reaction time was required (5hrs.) and some disubstitution was observed on tlc. However, problems were encountered during purification of the resulting monomer **[43]**. The compound appeared to be unstable during flash chromatography, reacting to give a side-product. Attempts to identify this were unsuccessful: the single signal observed on ^{31}P-NMR (δ -10) was outside the normal range for phosphotriester compounds (δ -4.0 to -6.0); the ^{1}H-NMR was dominated by the cholesterol moiety signals; and the mass spectrum contained signals which did not correlate to any obvious side-product. Only a moderate yield (57%) of the correct material, again a white solid, was recovered. It

was coupled onto the 5'-hydroxyl group of a dT 15mer sequence in anhydrous THF/doubly distilled pyridine (9:1) in the standard concentration (0.14mmol, 115mg). However, the measured coupling efficiency was poor (32%). Another attempt involving mild heating (50^{o}C for half an hour) resulted in no real improvement (36% coupling efficiency). The side product did not couple whatsoever when treated under the same conditions. Attempts to repeat the phosphorylation reaction resulted in even poorer yields and lower purity. When attempts were made to purify the phosphotriester monomer **[43]** using an alumina or cellulose column the same result was obtained. The coupling efficiency of the unpurified monomer **[43]** was low (25%) as would be expected.

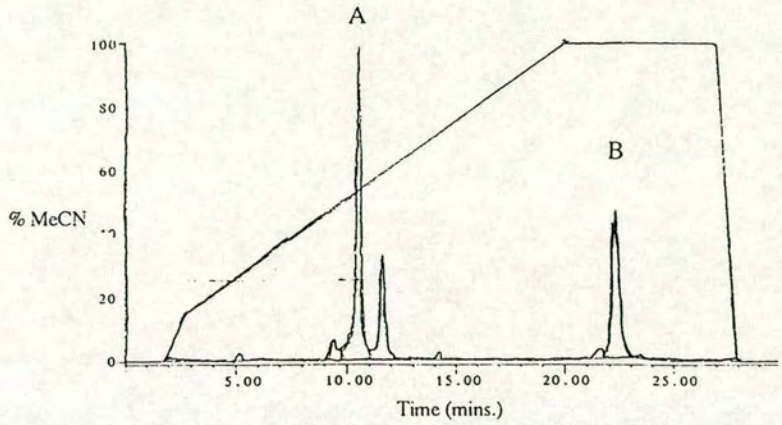
A curious solvent effect was observed during these studies; when the anhydrous THF content of the solvent mixture during the phosphotriester coupling procedure was replaced with anhydrous dichloromethane in which the cholesterol monomers **[42]** and **[43]** also dissolved, the coupling reaction was completely inhibited. This occurrence was independent of the monomers as shown by carrying out the coupling procedure in the absence of any monomer but with the other conditions as normal (123mg MSNT, 10% pyridine). When anhydrous THF was the solvent, no blocking of the available 5'-hydroxyl groups on the test oligonucleotide was observed (when the synthesis column was returned to the DNA synthesiser, a coupling efficiency of 98% was obtained for the addition of a standard dT residue). With the use of anhydrous dichloromethane as the solvent in the identical reaction, the opposite result occurred and no further addition onto the oligonucleotide was possible.

As a comparison to the phosphotriester cholesterol monomers, the phosphoramidite aminohexyl spaced cholesterol monomer **[3]** (p24, Chol-C6) was synthesised and coupled onto two dT 5mer oligonucleotides, one with and one without a 5'-terminal disulphide unit attached. The same extended phosphoramidite coupling cycle with anhydrous dichloromethane as solvent was used as before. Again, coupling onto the unmodified oligonucleotide was at a 93% coupling efficiency.

However, although slightly reduced from this, the coupling efficiency onto the 5'-disulphide group (83%) was still considerably higher than any coupling efficiency obtained using the cholesterol phosphotriester monomers above.

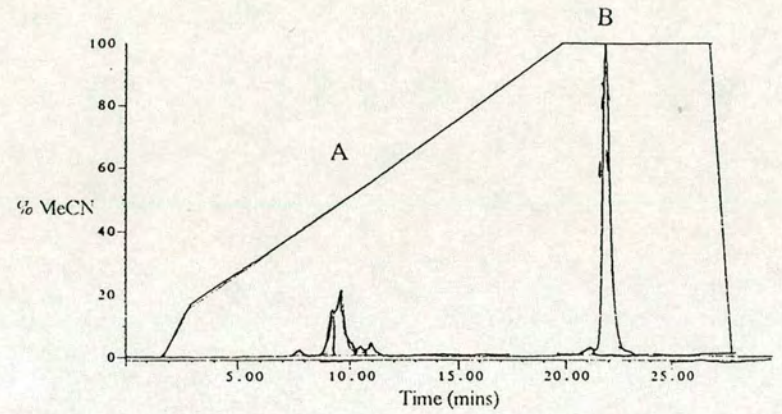
These values were confirmed by HPLC analysis. The chromatogram for the best of the phosphotriester cholesterol couplings onto a dT 5mer bearing the disulphide unit (***Figure 2.28***, Gradient **1**, p120) clearly showed a far higher failure sequence content (peak A) and far less product (peak B) then the trace for the phosphoramidite cholesterol monomer **[3]** coupled onto a dT 5mer bearing the terminal dipropyldisulphide group (***Figure 2.29***).

To prove its identity, the product from ***Figure 2.29*** (peak B) was collected and treated with DTT in the standard procedure. Gel filtration was not carried out as this would cause the loss of the oligonucleotide (only five residues in length). HPLC analysis was carried out on the product (***Figure 2.30***), revealing virtually quantitative disulphide cleavage to give peaks C and D.



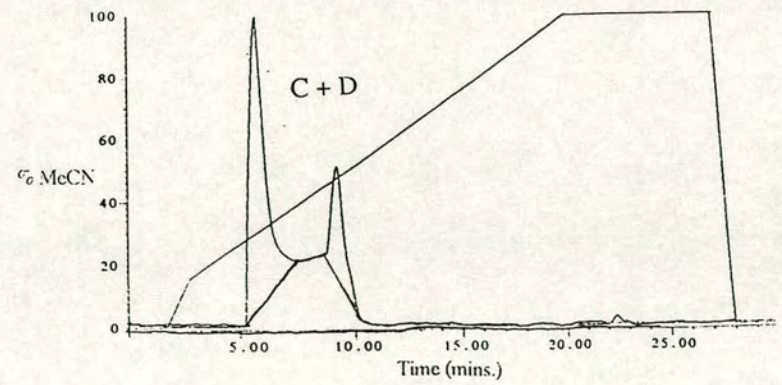
A : Failure Sequences B : Chol-SS-dT5

Figure 2.28 : Chol + SS-dT5 (Phosphotriester Coupling)



A : Failure Sequences B : Chol-C6-SS-dT5

Figure 2.29 : Chol-C6 + SS-dT5 (Phosphoramidite Coupling)

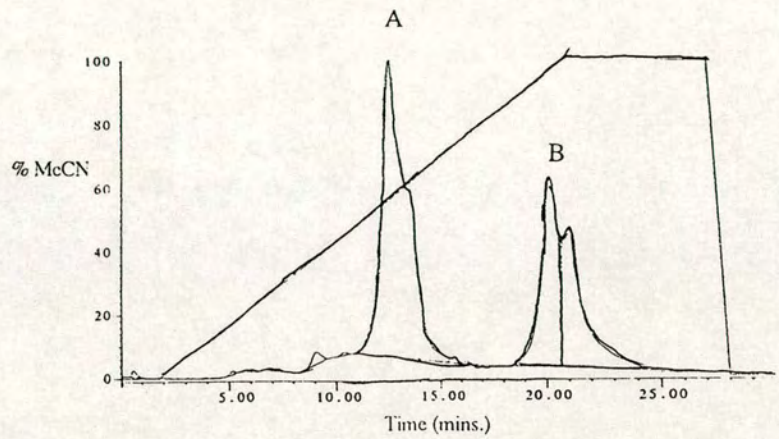


C + D : Excess DTT + Cleavage Products

Figure 2.30 : (Chol-C6-SS-dT5) + DTT

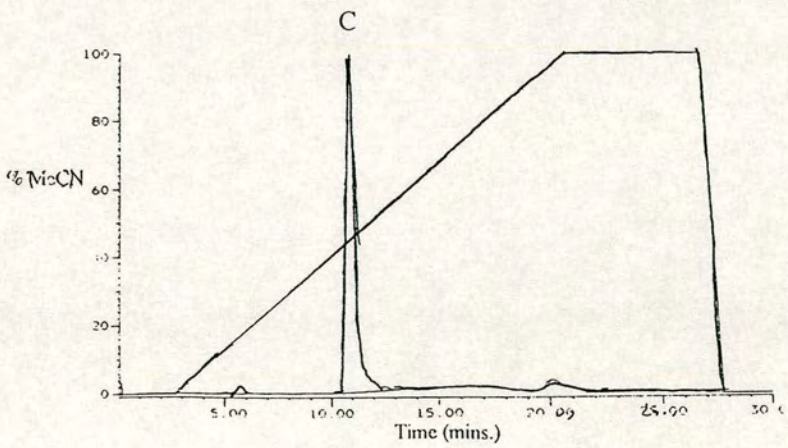
With a viable route to disulphide linked cholesterol oligonucleotides now established, it was decided not to pursue other synthetic methods. Interest in synthesising the molecules **[37]** (p55) and **[39]** (p56) with the lipophilic group and disulphide unit in the same molecule and converting them into phosphotriester monomers was set aside.

The phosphorothioate oligonucleotide, SRev, was synthesised once again and the phosphotriester disulphide monomer **[41]** (p59) was coupled onto its 5'-terminus using the standard non-automated procedure. Upon returning the synthesis column to the DNA synthesiser, capping and detritylation steps were carried out and the aminohexyl spaced cholesterol monomer **[3]** was added. The HPLC trace for the purified product is shown (***Figure 2.31***, Gradient **1**). The use of TETD for the sulphurisation step following the addition of monomer **[3]** could have resulted in some cleavage of the disulphide bond, causing the slightly reduced yield observed on HPLC analysis. The double peaks observed on both the failure sequences (peak A) and the product oligonucleotide (peak B) are a common feature of phosphorothioates bearing 5'-hydrophobic groups due to the interaction of the hydrophobic groups with the R/S isomer mix at phosphorothioate phosphorus atoms. Again, DTT treatment successfully cleaved the disulphide bond (***Figure 2.32***), with the single peak (C) obtained after gel filtration. The cleaved product (peak C) reacted with Ellman's reagent while the starting material (B) did not.



A : Failure Sequences B : Chol-C6-SS-SRev

Figure 2.31 : Chol-C6 + SS + SRev



C : HS-SRev

Figure 2.32 : (Chol-C6-SS-SRev) + DTT

Thus the potential anti-viral oligonucleotide, SRev, was synthesised bearing a cleavable cholesterol group. The synthesis procedure involved both phosphoramidite and phosphotriester chemistry with every coupling of high yield. This offers an alternative approach to the only other existing method for the synthesis of this type of modified oligonucleotide. Although not quoted, the yields from the published approach[112] are likely to be lower as H-phosphonate chemistry was used throughout and generally H-phosphonate coupling efficiencies are only ~95%, much worse than those obtained from either phosphoramidite (98-99%) or phosphotriester (~97%) chemistry.

If cellular uptake tests on the cleavable, cholesterol linked SRev oligonucleotide show promise, other lipophilic groups such as hexadecyl or 1,2-di-O-hexadecylglyceryl **[4]** (p45) can be added via a cleavable disulphide linker to SRev or any other oligonucleotide in an analogous manner.

2.1.3.6 Phosphorothioate Disulphide Monomers

A weakness in the cleavable cholesterol SRev oligonucleotide synthesised above is the single phosphodiester linkage between the disulphide group and the oligonucleotide. As cellular degradation of oligonucleotides occurs principally by the action of 3'-exonucleases and the unnatural 5'-monomer is unlikely to be a substrate for DNase nucleases, the significance of this weakness may be minimal. However, a means was sought to introduce a phosphorothioate linkage at this site.

The basic phosphorothioate building block, 2,5-dichlorophenyl-O,O-bis[(6-trifluoromethyl)-1-benzotriazoyl]phosphorothioate **[44]** was synthesised by the standard method and dissolved to give a 0.2M solution in dioxane.[74,75] This was reacted with the alcohol function of the disulphide unit **[31]** (***Figure 2.33***).

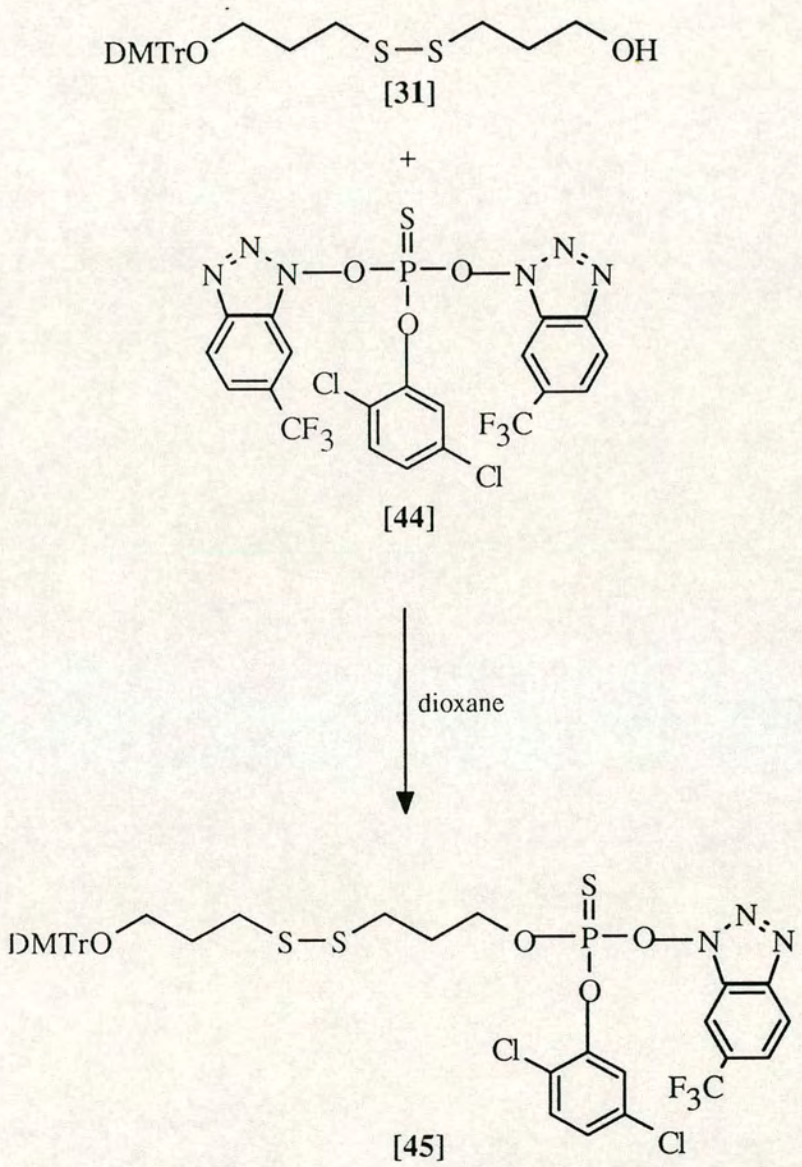


Figure 2.33 : Phosphorothioate Disulphide Monomer

In the method of de Vroom *et* al,[74,75] the whole coupling procedure was carried out in a one-pot, solution phase reaction. However, it was decided to isolate the product of the above reaction, [**45**], in order to enable solid phase addition of the monomer **[45]** according to the protocol adopted above. Two clear peaks were obtained on the ^{31}P-NMR spectrum of [**45**] (***Figure 2.34***), consistent with the expected R/S mixed stereochemistry at the phosphorus atom. The exact ratio between the peaks varied considerably between individual syntheses but the signal of higher δ value (55.03) was always of greater intensity, a phenomenon observed by others.[72]

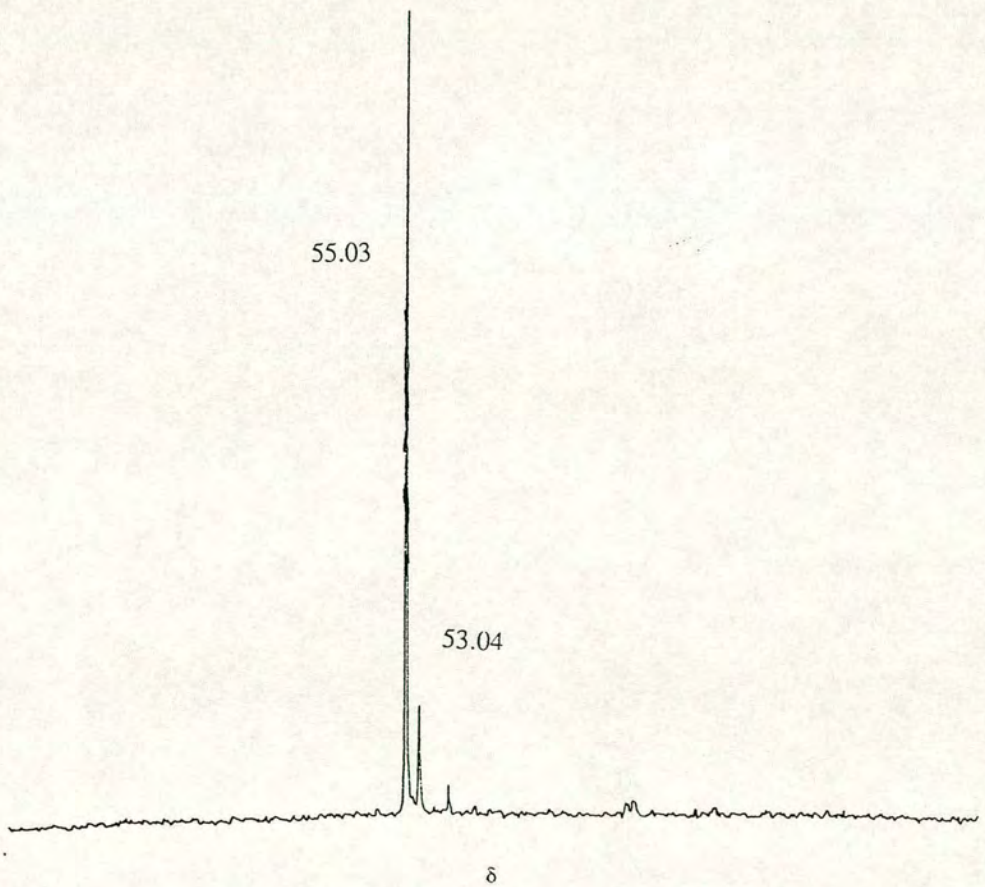


Figure 2.34 : ^{31}P-NMR of [45]

A short dT 5mer sequence was synthesised and coupling was attempted with the monomer **[45]**. The same conditions used for the disulphide monomer **[41]** were initially adopted – 0.14mmol (128mg) of monomer, anhydrous acetonitrile/doubly distilled pyridine (9:1, 0.20ml), 90 minutes coupling time – except for the absence of MSNT. Success was limited (20% coupling efficiency from trityl analysis) and subsequent increases in monomer concentration and coupling time resulted in little change (***Table 2.3***). The addition of N-methylimidazole into the solution mixture again resulted in complete inhibition of the reaction.

Table 2.3

Monomer (mmol)	0.14	0.30	0.42
Coupling Time (hrs.)	1.5	2.5	16
Coupling Efficiency (%)	20	20	26

The monomer **[45]** did not couple well under any of the conditions attempted. As a result, the cleavable, cholesterol linked SRev oligonucleotide was not synthesised as a full phosphorothioate. All previous attempts at synthesising phosphotriester phosphorothioates have been in solution phase only[72-75] with dimers formed in one-pot procedures. Perhaps this approach was not compatible with solid phase addition involving separate isolation of the monomers. However, no further investigations were carried out into the possibility of adapting the above work to solution phase synthesis.

(For a complete list of the oligonucleotides synthesised for anti-HIV testing, see **Appendix**).

2.2 Oligonucleotides Containing 5'-Thiols

Thiol groups at the 5'-terminus of oligonucleotides offer a simple and effective means for the attachment of thiol specific probes and reporter groups such as fluorescent dyes.[124-126,134] In addition, they can be used for the coupling of peptides and proteins to oligonucleotides under mild chemical conditions.[124,125] Literature routes to 5'-terminal thiols involve the addition of trityl protected monomers,[134,135,137] or a reaction of a thiol bearing small molecule with a 5'-amino functionalised oligonucleotide.[131,138] The disulphide monomer **[41]** (p59) offers an alternative route which has a number of advantages over the existing methods. Firstly, no amino functionalisation of the oligonucleotide is required prior to addition. More importantly, the monomer can be added during solid phase oligonucleotide assembly. Deprotection to give the propylthiol is achieved in a simple process involving DTT with no extra step such as the addition of silver ions required. In addition, before cleavage, the thiol is constantly protected against dimerisation. The DMTr group permits the measurement of the coupling efficiency of the monomer which has been found to be consistently high yielding. Alternatively, if left on, it offers a means to assist purification during HPLC analysis in which the product oligonucleotide elutes much later than the failure sequences. In addition, the molecule can be introduced selectively at any site in an oligonucleotide sequence; DTT treatment always produces the terminal thiol group.

To confirm that the product of DTT cleavage of an oligonucleotide containing the disulphide unit is a thiol, the oligonucleotide resulting from cleavage was treated with the thiol specific label, 5-iodoacetamidofluorescein (IAF)[139] **[18]** (p35, ***Figure 2.35***), for 5 hours at room temperature in DMF. On this occasion, the oligonucleotide chosen was an 18mer of mixed sequence : d(ATG GCT CTC CCG GGA GGG).

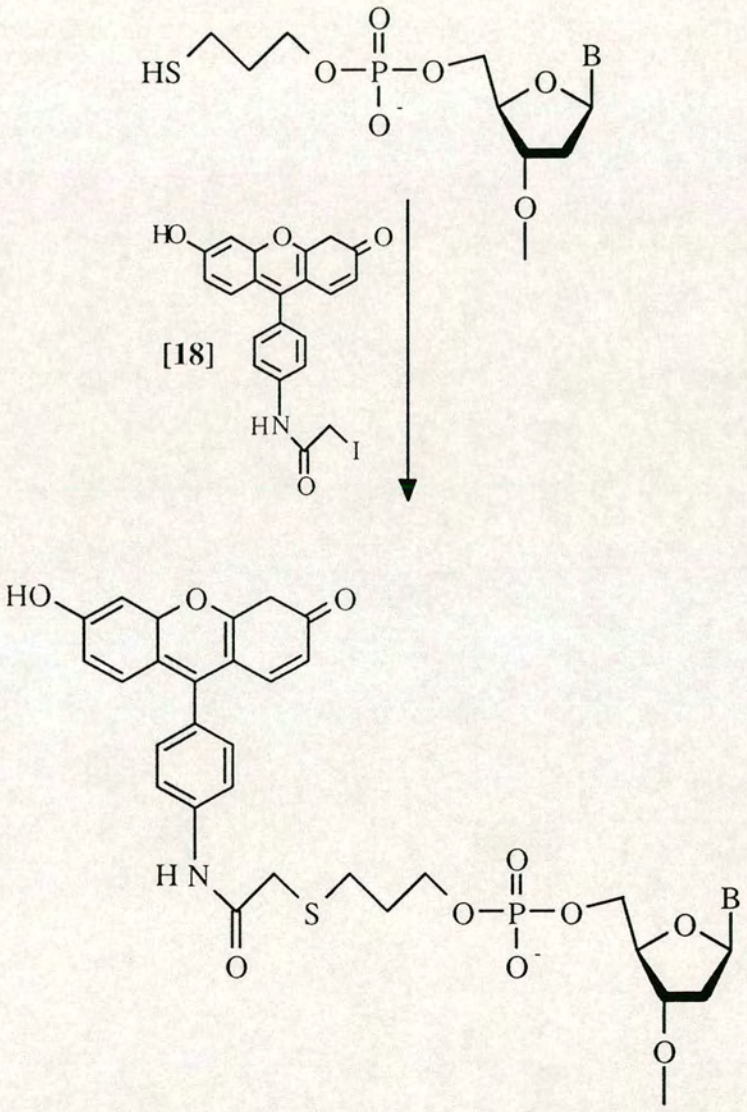
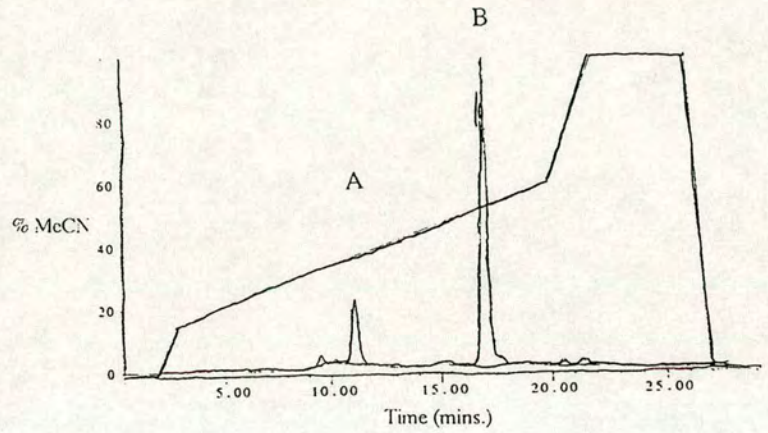


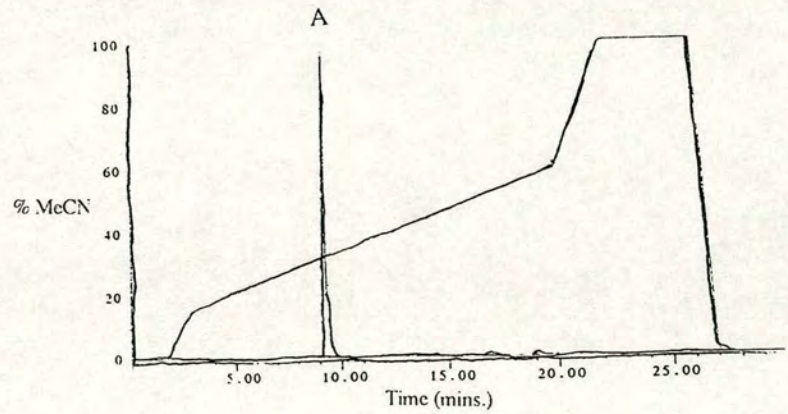
Figure 2.35 : 5-Iodoacetamidofluorescein Reaction

Following repeated gel filtration steps to ensure the complete removal of unreacted IAF molecules, the product was analysed on reverse phase HPLC (***Figure 2.36***, Gradient **2**, p120) and compared to the 5'-terminal propylthiol monomer (***Figure 2.37***) which had been purified prior to DTT cleavage (i.e. while still carrying a DMTr group) and therefore separated from its failure sequences. The peak representing the thiol functionalised oligonucleotide (A) diminished and the product of the reaction with IAF appeared (B).



A : HS-18mer B : IAF-S-18mer

Figure 2.36 : HS-18mer + IAF



A : HS-18mer

Figure 2.37 : (SS + 18mer) + DTT

The ratio of product to starting material (approximately 5:1) shows how well the reaction had gone. No reaction occurred between IAF and an uncleaved disulphide containing oligonucleotide, nor was there any reaction between the failure sequences from the 5'-terminal disulphide 18mer synthesis and IAF; therefore proving that no disulphide reduction occurs during deprotection at all.

This offered a more conclusive method for testing the product of disulphide cleavage than Ellman's reagent (***Figure 2.38***) which undergoes disulphide cleavage in the presence of thiols to give a bright yellow colour. Ellman's reagent offers a quicker but less reliable test – it also reacts with P(III) containing compounds – although it has been used successfully a number of times during this work.

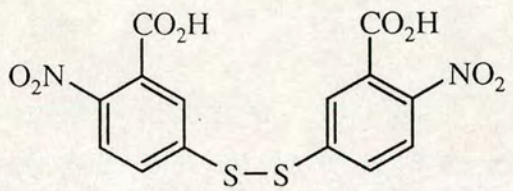


Figure 2.38 : Ellman's Reagent

The disulphide unit **[31]** (p82), as has already been shown, can be introduced at the 3'-terminus of an oligonucleotide. Thus a route is also available for the synthesis of 3'-terminal thiols. However, as several methods already exist for the synthesis of such oligonucleotides using similar chemistry,[124-127] this is less significant than the method developed for the synthesis of 5'-thiols using the monomer **[41]**.

2.3 Oligonucleotides Containing 5'-Phosphates

The synthesis of oligonucleotides by the standard methods yield a product with a free 5'-hydroxyl group after deprotection. However, many biological processes such as total gene synthesis, chemical ligation or structural studies require oligonucleotides bearing a 5'-phosphate group. It has already been shown that the product of the cleavage of the disulphide group of monomer **[41]** when contained in an oligonucleotide was a propylthiol. It was decided to use the same general methodology to produce a monomer which results in a phosphate group at the oligonucleotide terminus following disulphide reduction. It has been observed on a number of occasions[125,126,136] that if the alkyl chain attached to a disulphide unit were only two carbons in length, the thiol which results from disulphide cleavage will, under basic conditions, give a phosphate by a β-elimination mechanism (***Figure 1.18***, p29). For that reason, such a monomer was synthesised (***Figure 2.39***).

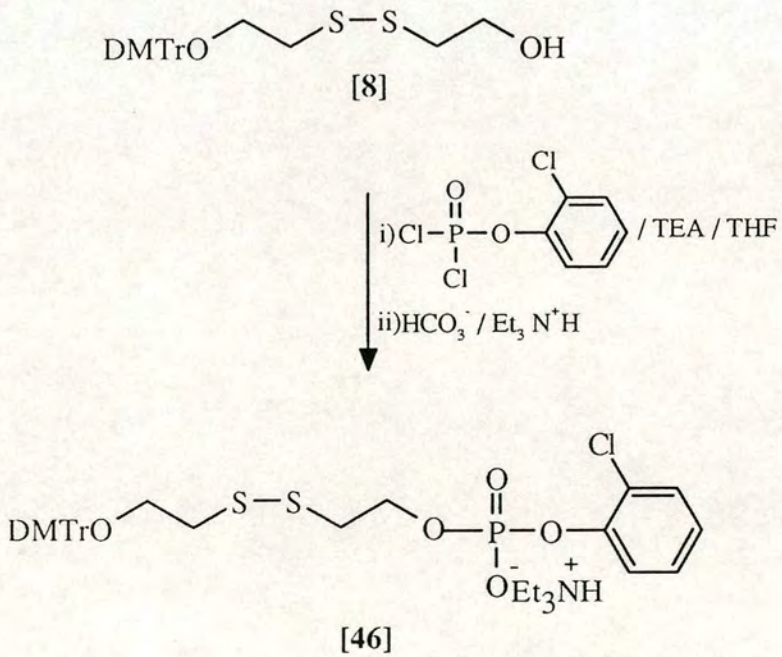
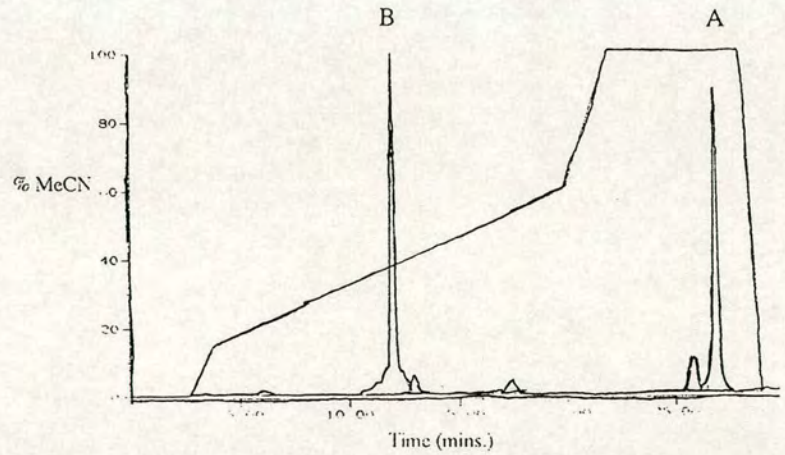


Figure 2.39 : C2 Disulphide Monomer

The disulphide containing compound **[8]** was synthesised according to the procedure of Asseline *et al*[126] from dithiodiethan-1,1'-ol. Phosphorylation was carried out in THF using 2-chlorophenyldichlorophosphate with the addition of 1.0M TEAB solution after 2 hours.

The ^{31}P-NMR spectrum showed only one product from the reaction and when coupling was attempted with the monomer **[46]** (C2SS) onto a dT sequence 12 nucleotides in length using the standard protocol (0.14mmol, 104mg of monomer in acetonitrile/pyridine) the coupling efficiency was measured at 94%. However, when another 2'-deoxythymidine residue was coupled onto the disulphide, the resulting oligonucleotide with the 5'-DMTr group still attached but the *o*-chlorophenyl group removed, gave a trace on reverse phase HPLC (***Figure 2.40***, Gradient **2**, p120) which seemed to suggest that a far lower coupling efficiency had occurred. Even a poor coupling onto the disulphide group by the phosphoramidite monomer could not account for it.



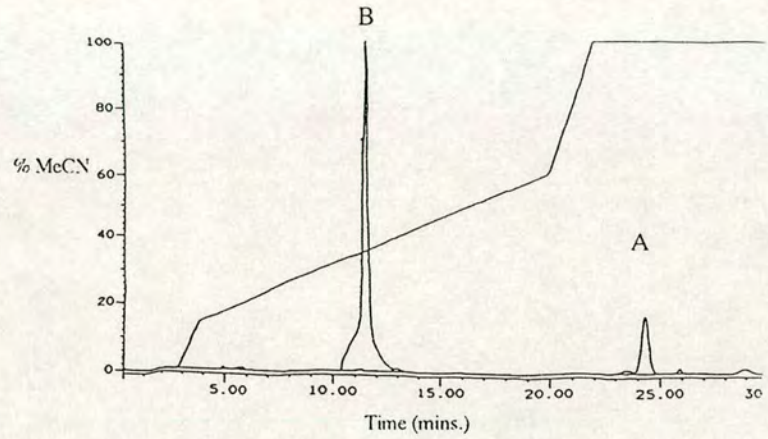
A : C2SS-dT12 B : Failure Sequces

Figure 2.40 : C2SS + dT12

Further phosphorylation reactions were attempted but the same result was obtained although the exact ratio between the peaks of the product and the failure sequences was variable. Why the coupling efficiency values calculated always indicated a normal coupling of 94-95% while the evidence of the HPLC traces was apparently contradictory was uncertain. Successful DTT cleavage of the product (peak A) to give a peak which eluted at a time similar to that of the failure sequences (peak B) and gave a positive result with Ellman's reagent confirmed that the expected oligonucleotide had indeed been synthesised. It was initially thought that perhaps the poor couplings were for some reason a feature of this particular monomer but when the same result was obtained for a subsequent synthesis of the monomer **[41]** (p59), it seemed to suggest that there was a problem with the coupling procedure itself. The double distillation of the pyridine was repeated, the MSNT was analysed by ^{1}H-NMR and mass spectrometry to confirm that its purity was high and modifications to the coupling procedure were attempted, all without avail. A sample treated at 55^{o}C for two days in ammonium hydroxide gave the identical HPLC trace to that after 5 hours at 55^{o}C; confirming that the monomer is stable to the deprotection conditions and no post-synthetic decomposition was occurring. Curiously, when mixed base sequence oligonucleotides of 15-20 residues in length were reacted with the disulphide monomer, upon HPLC analysis, even lower quantities of product were observed. This was independent of whether the 5'-nucleotide was dT, dC, dA or dG.

It was only when the ^{1}H-NMR spectrum for a sample of the monomer **[46]** was examined more closely that a possible answer was discovered. The relative size of the TEA signals in the spectrum were greater than expected for a simple triethylammonium salt. Clearly, if there was any TEA present in the coupling solution, it would have an inhibitive effect on the coupling reaction. When the monomer **[46]** was coupled onto a dT 15mer sequence once again, the reaction was carried out with double the quantity of monomer. The disulphide unit was not detritylated and HPLC analysis of the product (***Figure 2.41***, Gradient **2**) following

deprotection gave an even poorer yield. Increasing the quantity of the monomer should have little effect on the coupling efficiency by itself, but if the TEA content of the monomer solution was doubled correspondingly, the yield would be reduced as indeed was observed.



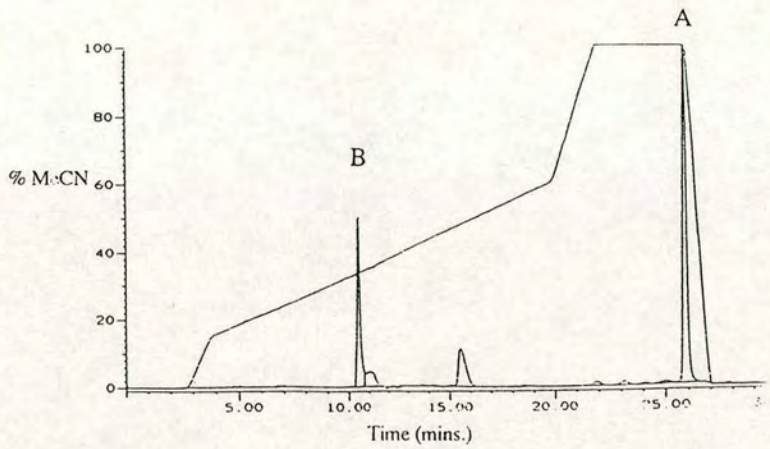
A : C2SS-dT12 B : Failure sequences

Figure 2.41 : (2 x C2SS) + dT15

Triethylamine is a necessary component in the silica-gel flash column chromatography purification of the phosphotriester monomers in order to maintain the monomers as their triethylammonium salts. In this way, the exact TEA content of the monomers would vary thus accounting for the erratic nature of the HPLC traces being obtained.

Until this problem arose, TEA was removed from the purified phosphotriester monomers by overnight drying of the monomers in a vacuum desiccator. The phosphorus pentoxide drying agent in the desiccator was replaced with fresh drying agent and a sample of the phosphotriester monomer **[46]** was subjected to prolonged vacuum desiccation. A standard coupling reaction onto a dT 15mer oligonucleotide was performed with the re-dried sample and the HPLC analysis of the product oligonucleotide with the DMTr group still attached and the *o*-chlorophenyl group

removed (*Figure 2.42*, Gradient 2) confirmed a favourable ratio of product to failure sequence peaks, as would be expected from the coupling efficiency values obtained.



A : C2SS-dT15 B : Failure sequences

Figure 2.42 : C2SS + dT15

When the sample of monomer **[41]** which had previously been giving low coupling efficiency was subjected to the same drying treatment, the HPLC analysis once again indicated a high coupling efficiency. In addition, the coupling of monomer **[46]** onto a mixed base sequence proceeded smoothly (HPLC analysis).

Every phosphotriester monomer used henceforth was subjected to careful drying prior to use. There was a possibility that the cholesterol containing phosphotriester monomers **[42]** (p74) and **[43]** (p75) prepared previously were contaminated with TEA. This would account for the very poor coupling efficiencies obtained for these monomers. However, in these cases, the monomers were in the form of solids thereby easier to dry. Also, elemental analyses of these molecules were close to the expected C, H and N content. This therefore seems unlikely.

The reported protocol to reduce the disulphide group in an oligonucleotide to a phosphate varies widely, from 90 minutes at room temperature[136] to 6 hours at

55°C[126] or 16 hours at 55°C,[125] all using DTT in ammonium hydroxide. The key factor is the rate of mercaptoethanol elimination once the disulphide has been reduced to the thiol. Is prolonged heating required or does the elimination reaction occur spontaneously at room temperature?

The dT 15mer with the disulphide monomer **[46]** coupled to its 5'-terminus (peak A, ***Figure 2.42***) was treated with DTT in ammonium hydroxide both for 1 hour at room temperature – the standard disulphide cleavage conditions – and for 3 hours at 65°C. Subsequent HPLC analysis of the gel filtered products (***Figures 2.43, 2.44***, Gradient 2) showed little difference between the samples. A dT 15mer sequence was synthesised with the addition of the standard phosphate monomer **[19]** (p35) onto its 5'-terminus. The resulting 5'-terminal phosphate (***Figure 2.45***) was run as a mixed sample with the two products of DTT treatment (***Figure 2.46***). The unambiguous single peak obtained seemed to indicate that the identity of all three oligonucleotides was the same.

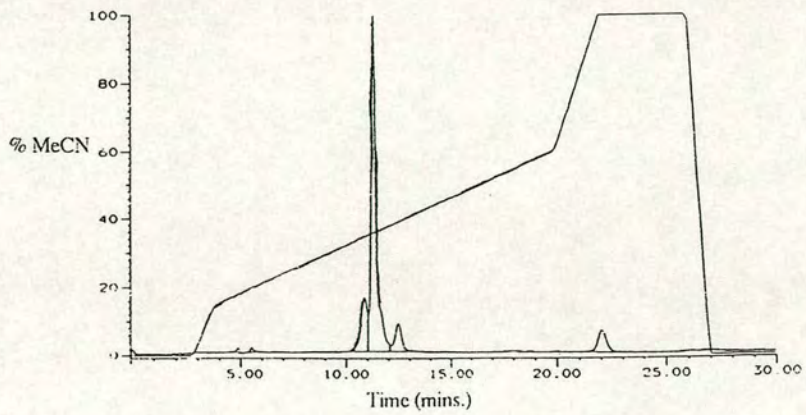


Figure 2.43 : (C2SS-dT15) + DTT (1 hour)

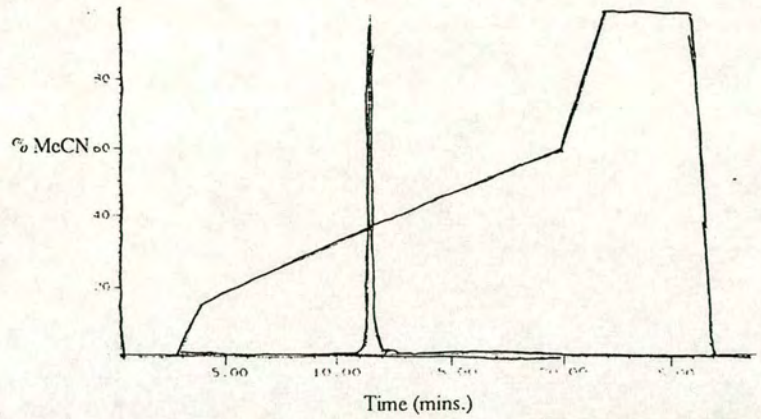


Figure 2.44 : (C2SS-dT15) + DTT (3 hours)

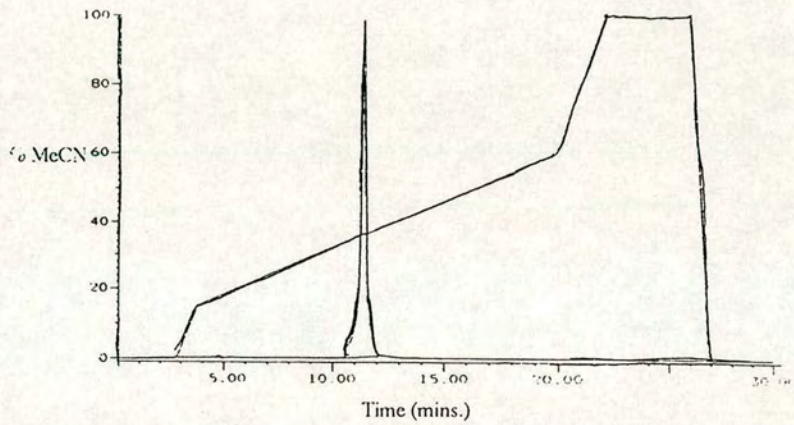


Figure 2.45 : [19] + dT15

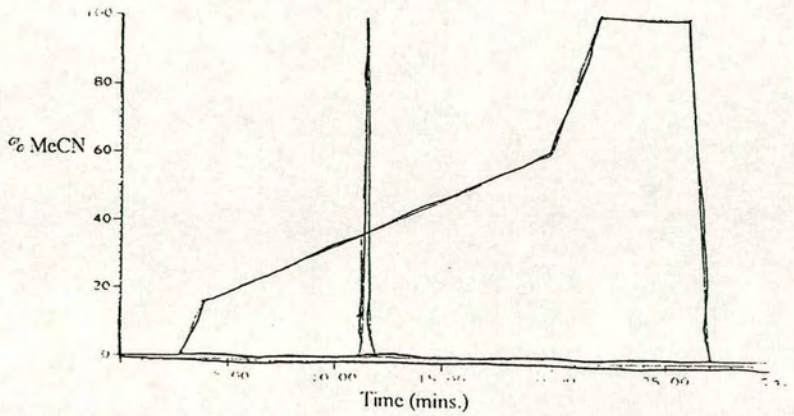
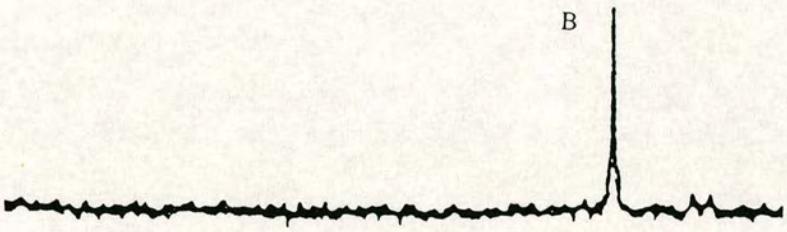


Figure 2.46 : Mixed Sample

However, it has already been observed that reverse phase HPLC is not always capable of separating oligonucleotides with a small difference in lipophilicity, for example with and without an alkyl chain of only two carbons in length. Capilliary zone electrophoresis (CZE) separates oligonucleotides based on their charge content in addition to their lipophilicity. The high sensitivity of this technique should be sufficient to distinguish between a 5'-terminal phosphate and a 5'-terminal thiol of an oligonucleotide of the same base sequence. The difference between the oligonucleotide still with the disulphide group intact and bearing a 5'-DMTr group (***Figure 2.47***, peak A) and the product of DTT treatment for 1 hour at room temperature (***Figure 2.48***, peak B) was clear. When the conventionally synthesised 5'-phosphate was analysed (***Figure 2.49***, peak C), the product peak had an identical elution time to the product of disulphide cleavage. A mixed CZE sample of all three of the above (***Figure 2.50***, A + B + C) showed the difference between the disulphide containing oligonucleotide which eluted last and the other two oligonucleotides which again were present as one unambiguous peak.

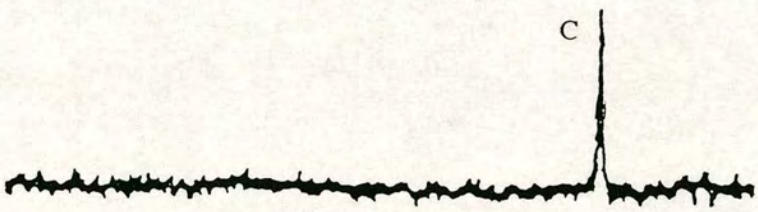
A : DMTr-dT15

Figure 2.47 : C2SS-dT15



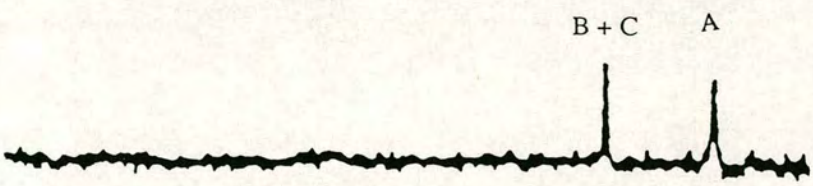
B : phosphate-dT15

Figure 2.48 : (C2SS-dT15) + DTT (1 hour)



C : phosphate-dT15

Figure 2.49 : [19] + dT15



A : DMTr-dT15 B + C : phosphate-dT15

Figure 2.50 : Mixed Sample

As a final experiment, it was decided to react the oligonucleotide obtained from the disulphide reduction reaction above with IAF. The procedure was identical to that used for reaction between IAF and the 5'-terminal thiol oligonucleotide obtained using monomer **[41]** (see Section **2.2**, p85). On this occasion, there was no evidence whatsoever for any reaction having occurred, showing that there was no thiol present at all in the sample. The HPLC trace of the product (***Figure 2.51***, Gradient **2**), following gel filtration, showed only one main peak which was the unreacted oligonucleotide.

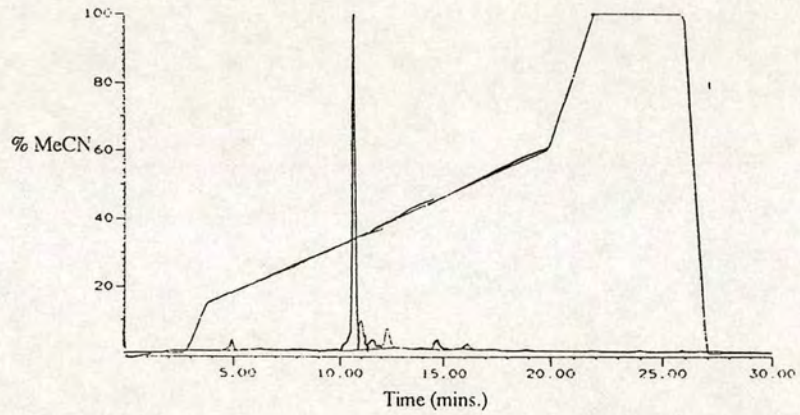


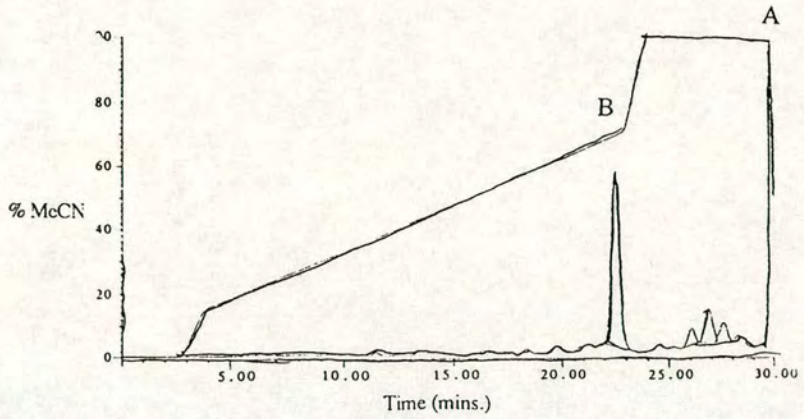
Figure 2.51 : (C2SS-dT15 + DTT) + IAF

From the evidence, it seemed extremely likely that the desired 5'-terminal phosphate was the product after the oligonucleotide bearing the disulphide unit **[8]** (p89) was treated with DTT in base. Thus an alternative to the phosphate monomer **[19]** (p35) has been developed, also added during solid phase oligonucleotide assembly but compatible with the phosphotriester coupling approach. In addition, the monomer **[46]** (p89) presents an alternative to the monomer **[41]** (p59) as a source of a cleavable linker to be coupled to the 5'-terminus of an oligonucleotide.

2.4 Oligonucleotides Containing a Cleavable Biotin

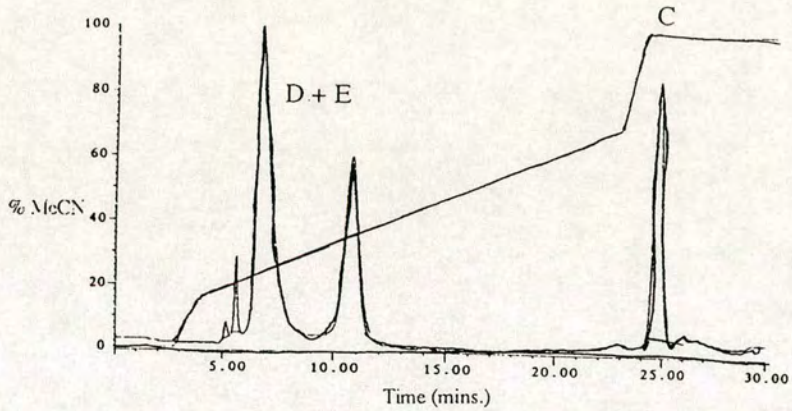
Oligonucleotides can be biotinylated prior to their application in the polymerase chain reaction (PCR) to enable the amplified products to be isolated (on streptavidin). However, the binding between the biotin and streptavidin is so tight ($K_d = 10^{-15}M$) that problems are encountered freeing the oligonucleotides. The only method currently available for the release of a biotin labelled oligonucleotide captured on streptavidin is to use the cleavable biotin 2'-deoxyuridine triphosphate **[15]** (***Figure 1.20***, p32),[132] developed from sulphosuccinimidyl-2-(biotinamido)ethyl-1,3'-dithiopropionate (NHS-SS-Biotin) **[14]** (p32). It contains a disulphide linker between the biotin and a 2'-deoxyuridine base which is cleaved by DTT. However, the synthesis of such a monomer involves a three step base modification procedure and the resulting modified 2'-deoxyuridine triphosphate can be incorporated into an oligonucleotide by the non-selective nick-translation technique or other enzymatic methods. The employment and extension of the disulphide phosphotriester chemistry developed above offers potentially superior methods for the incorporation of cleavable biotin molecules into oligonucleotides; the synthesis procedures are straightforward and the monomers can be added to the 5'-terminus of oligonucleotides during solid phase assembly.

The first method used for biotinylating an oligonucleotide via a cleavable linker was the straightforward coupling of the biotin phosphoramidite monomer **[24]** (***Figure 1.28***, p40) onto a disulphide functionalised oligonucleotide. A dT sequence 6 nucleotides in length was synthesised and the disulphide phosphotriester monomer **[41]** was added as before. Following capping and detritylation, the biotin monomer was added using a standard automated coupling cycle. The DMTr and *o*-chlorophenyl groups were removed and the product was analysed on reverse phase HPLC (***Figure 2.52***, Gradient **2**, p120). The target oligonucleotide (peak A) appeared as a larger peak than the failure sequences (peak B).



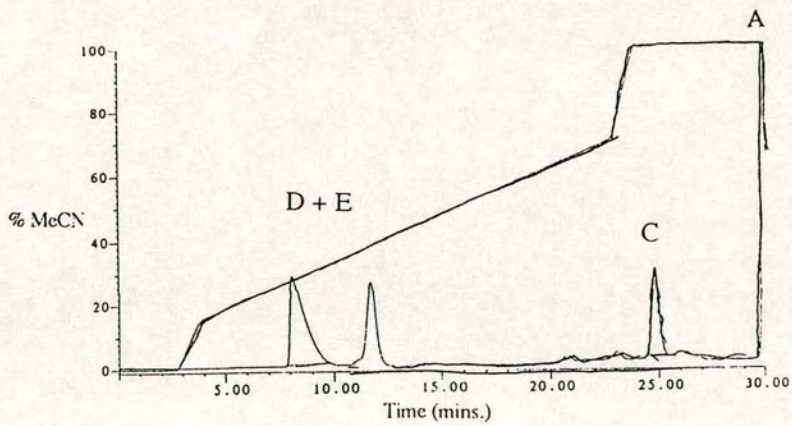
A : Biotin-SS-dT6 B : Failure Sequences

Figure 2.52 : Biotin + (SS + dT6)



C : HS-dT6 D + E : Excess DTT + Cleavage Products

Figure 2.53 : (Biotin-SS-dT6) + DTT



A : Biotin-SS-dT6 C : HS-dT6 D + E : Excess DTT + Failure Sequences

Figure 2.54 : Biotin-SS-dT6 + HS-dT6

Once isolated, the product oligonucleotide was treated with DTT, without any subsequent gel filtration step as it was only 6 nucleotides in length (***Figure 2.53***). The cleaved oligonucleotide (peak C) lay between the failure sequences (B) and the starting oligonucleotide (A). The residual DTT eluted far earlier (peaks D and E). An analysis of a mixed sample of the above (***Figure 2.54***) showed the difference between the original oligonucleotide (A) and the cleaved product (C). A positive result with Ellman's reagent confirmed that a thiol was the product of cleavage.

However, the coupling efficiency of the biotin phosphoramidite monomer (59%) was substantially reduced from standard (97%), even more than previously observed for a coupling onto a 5'-disulphide unit. Although the above method is still a valid synthetic route to cleavable biotin oligonucleotides, it was thought desirable to develop another method possessing potentially higher yields. A biotin phosphotriester monomer could have been synthesised but after the poor results of the two cholesterol derived phosphotriester monomers **[42]** (p74) and **[43]** (p75), it was decided to synthesise and phosphorylate a molecule containing both the biotin and disulphide groups.

Initially, an amino functionalised disulphide unit was synthesised (***Figure 2.55***) analogously to before (***Figure 2.8***, p54) with *t*-butyldimethylsilyl (TBDMS) as the hydroxyl protecting group to allow the DMTr protecting group to be used to protect the 1' position of the biotin molecule. The disulphide containing diol **[30]** was protected at a single hydroxyl group by reaction with one equivalent of *t*-butyldimethylsilyl chloride in pyridine. The available hydroxyl group of the product **[47]** was reacted with tosyl chloride in pyridine. During this step, compound **[47]** appeared to be far more hygroscopic than the analogous DMTr protected molecule **[33]** (p54) and so extra precautions involving careful distillation of the solvents and vacuum desiccation of **[47]** were taken to ensure anhydrous conditions and to avoid the oxidation of tosyl chloride to tosic acid. The tosylate group of **[48]** was then displaced by potassium phthalimide in DMF.

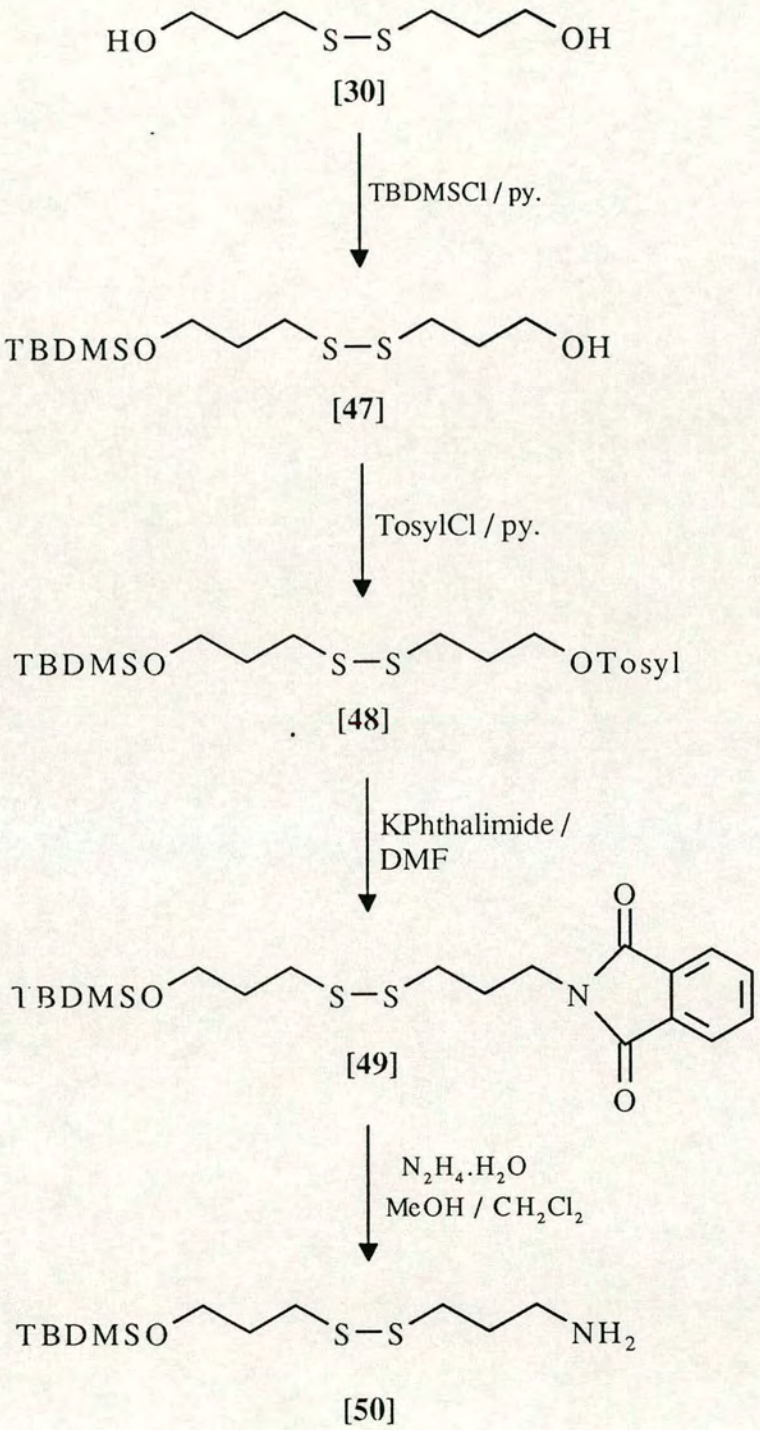


Figure 2.55 : TBDMS Amino Disulphides

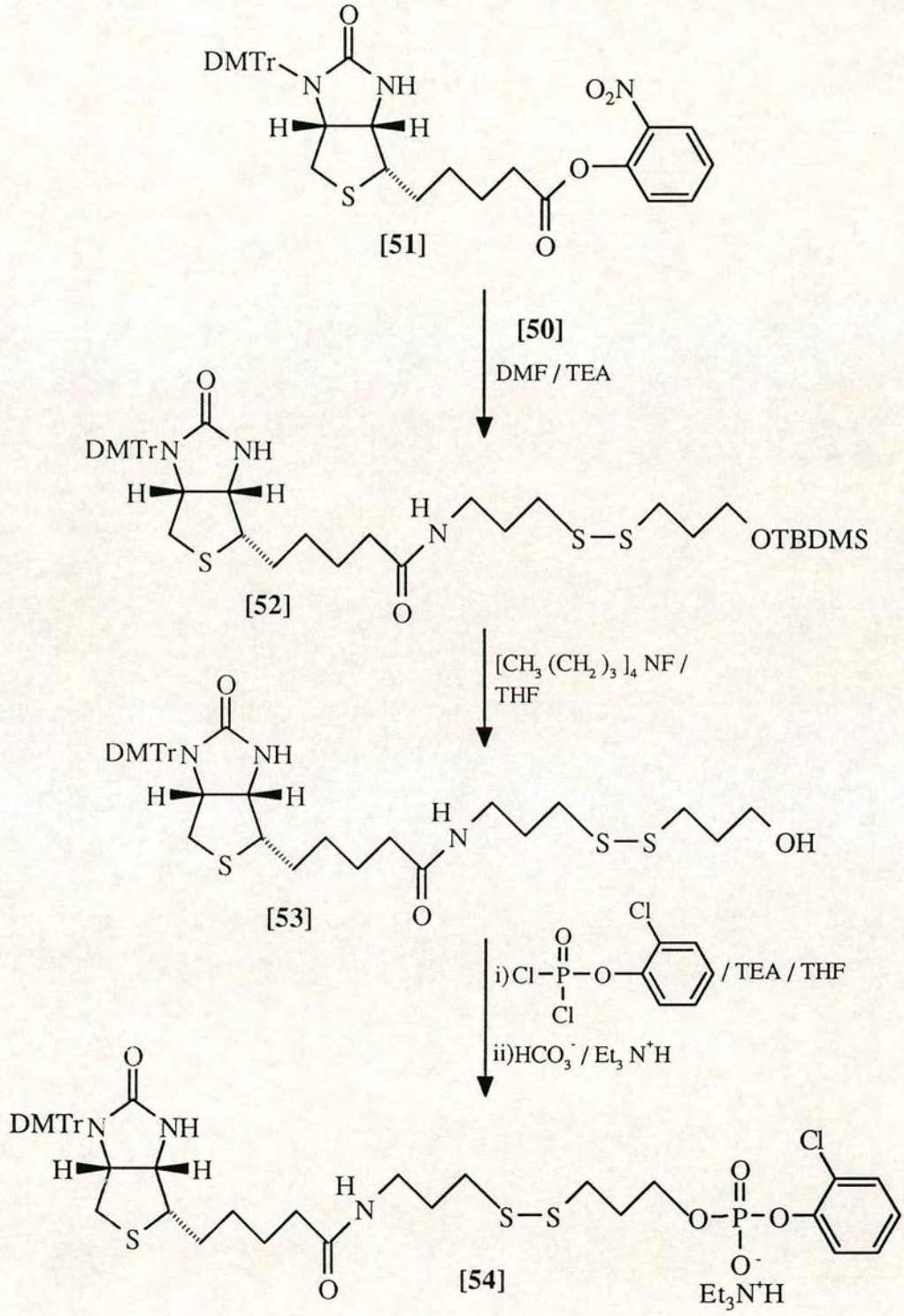
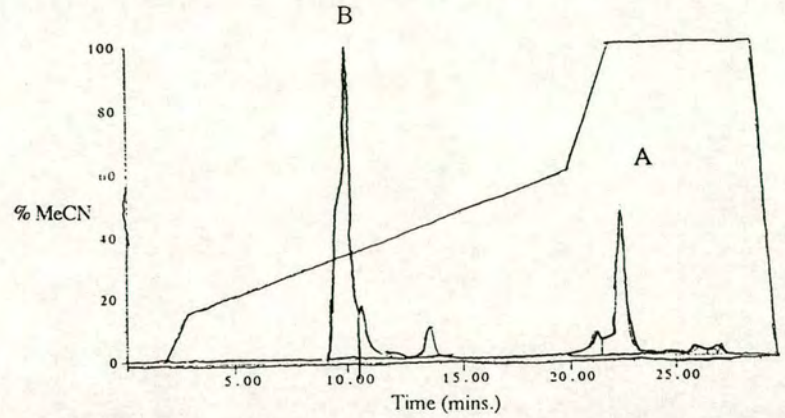


Figure 2.56 : Cleavable Biotin Monomer

Conversion of the phthalimide **[49]** to the amine **[50]** was carried out with hydrazine hydrate in a (1:1) dichloromethane/methanol mixture.

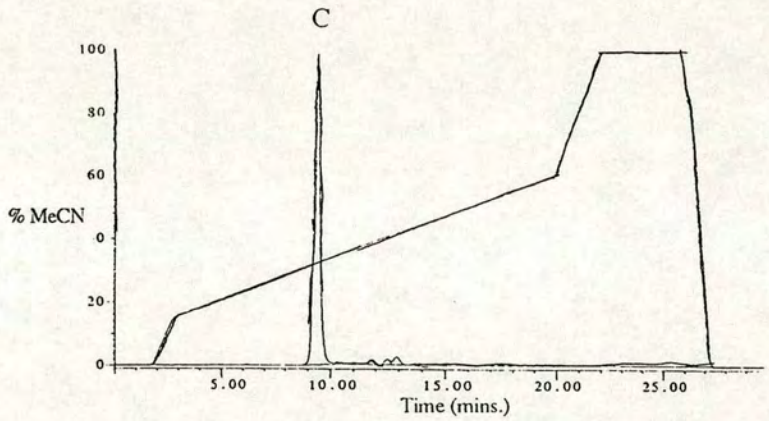
(+)Biotin-2-nitrophenylester was prepared by the procedure of Sproat *et al.*[157] The 1' position of the biotin ring was then protected by reaction with 4,4'-dimethoxytrityl chloride in pyridine. The reaction between the protected biotin active ester **[51]** and the disulphide amine **[50]** (***Figure 2.56***) in DMF in the presence of TEA proceeded only very slowly. The product **[52]** was isolated after 5 days. Removal of the silyl protecting group by treatment with tetrabutylammonium fluoride solution (1.0M in THF) yielded the compound **[53]**. However, phosphorylation to give the monomer **[54]** did not work as cleanly as in previous examples. The yield was only moderate (48%) and the ^{31}P-NMR spectrum contained only a weak signal and a high level of noise, even after precautions had been taken to remove all residual TEA present.

When the monomer (Biotin-SS) was added to a dT 15mer sequence in the standard non-automated procedure (0.14mmol, 140mg of monomer in acetonitrile/pyridine) a coupling efficiency of only 45% was obtained. This was reflected in the HPLC analysis (***Figure 2.57***, Gradient **2**) although when the product oligonucleotide (peak A) was treated with DTT, cleavage was once again quantitative to give an earlier eluting peak (C, ***Figure 2.58***), which reacted with Ellman's reagent.



A : Biotin-SS-dT15 B : Failure Sequences

Figure 2.57 : Biotin + dT15



C : HS-dT15

Figure 2.58 : (Biotin + dT15) + DTT

It seems likely that the relatively poor coupling efficiency of the monomer **[54]** was a reflection of the poor phosphorylation reaction, rather than the presence of any TEA. Nevertheless, the one addition reaction carried out indicated the potential usefulness of this route to a cleavable biotin monomer and if the procedure were optimised, it could prove extremely valuable. Furthermore, using the shorter C2 disulphide unit, a cleavable biotin monomer could be developed in which the product of the cleavage reaction was a 5'-terminal phosphate instead of a 5'-terminal thiol.

2.5 Oligonucleotides Containing a Cleavable Aminolinker

There is a great demand for the synthesis of oligonucleotides bearing a 5'-terminal amino functionality. The amino group is more reactive than the hydroxyl group and offers an easy means for the attachment of a variety of different labelling or reporter groups. A cleavable aminolink at the 5'-terminus of an oligonucleotide would enable the oligonucleotide to be detached from the labelling group once the labelling molecule was no longer required. As yet no cleavable aminolink monomers have been developed. The phosphotriester coupling chemistry described above can easily be extended to the synthesis of such a monomer, attachable during solid phase oligonucleotide assembly.

For the synthesis of the aminolink monomer, it was decided to use DMTr as the amine protecting group. When attached to primary amines, the DMTr group is far more labile than when used as a hydroxyl protecting group due to the increased propensity of the amine to protonation. However, if precautions are taken to speed up the purification procedure and to maintain a basic environment throughout, this type of compound can be handled, although with reduced reaction yields because of a certain amount of unavoidable detritylation. The advantage of using the DMTr group is that the coupling efficiency for the cleavable aminolink monomer can be accurately calculated instead of having to estimate it from HPLC analysis.

The TBDMS protected amino disulphide molecule **[50]** was reacted with 4,4'-dimethoxytrityl chloride in pyridine (***Figure 2.59***). The product **[55]** was deprotected at the hydroxyl group by treatment with tetrabutylammonium fluoride solution (1.0M in THF) to give compound **[56]**. This was then phosphorylated in THF with 2-chlorophenyldichlorophosphate to give the phosphotriester monomer **[57]**.

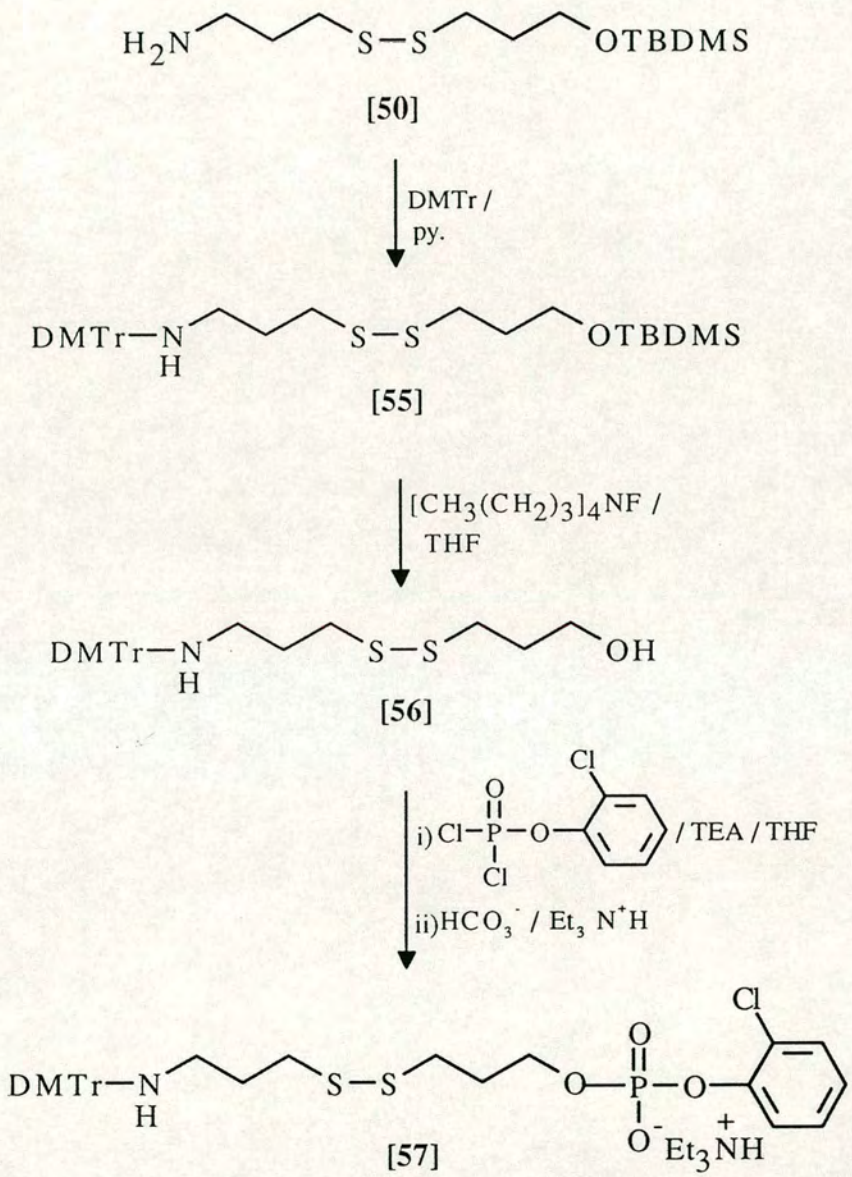


Figure 2.59 : Cleavable Aminolink Monomer

As expected, the yields for each step in the reaction scheme were relatively poor due to detritylation. However, the phosphotriester monomer which resulted **[57]** had a promising ^{31}P-NMR spectrum and when coupling was attempted after removal of any residual TEA (0.14mmol, 109mg of monomer in acetonitrile/pyridine) onto a detritylated dT 15mer sequence, the measured coupling efficiency for the monomer was extremely encouraging (85%). Although the absence of a protecting group made the product oligonucleotide indistinguishable from the failure sequences during HPLC analysis (***Figure 2.60***, Gradient **3**, p120) and DTT treatment follwed by gel filtration gave a peak with the identical elution time (***Figure 2.61***), testing with the amino sensitive ninhydrin spray was more revealing. The oligonucleotide with the intact disulphide aminolink turned a dark blue colour when sprayed with ninhydrin on a tlc plate but the product of the cleavage reaction gave no reaction under the same conditions.

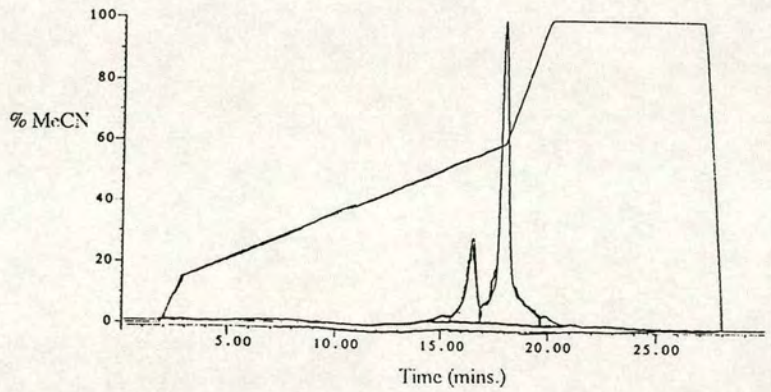


Figure 2.60 : [57] + dT15

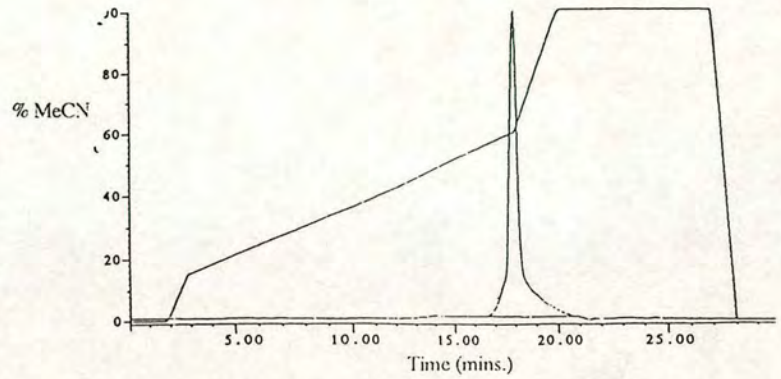


Figure 2.61 : ([57]-dT15) + DTT

Only once was coupling attempted but it showed considerable promise. If the coupling procedure is optimised, it is likely that even higher coupling efficiencies will be possible. The DMTr group used in these initial studies enabled the coupling efficiency of the monomer to be measured but as a protecting group it was barely adequate. For the future development of this cleavable aminolink, a more stable protecting group such as 4-monomethoxytrityl (MMTr) or trifluoroacetyl would have to be used. In particular, the ABI 394 DNA synthesiser measures trityl yields by conductivity, treating DMTr and MMTr equally and it is therefore possible to accurately measure MMTr coupling efficiencies on this machine. As amino functionalised oligonucleotides are often used for biotinylation,[146,151-154] this cleavable aminolink monomer offers a third route to cleavable biotin containing oligonucleotides, a possibility which could be investigated, along with its reaction with other amino specific labels. In addition, the TBDMS protected molecule **[56]**, an intermediate in the synthesis of both the cleavable biotin and cleavable aminolink monomers, **[54]** and **[57]**, could also have been used to synthesise the cleavable lipophilic molecules **[37]** (p55) and **[39]** (p56) with subsequent phosphorylation.

Clearly, however, the described route to cleavable aminolink monomers, with the disulphide group as the cleavable entity, has been shown to have merit. Once a monomer is developed, the alternative C2 linked disulphide unit could be treated in a manner analogous to the C3 disulphide unit; the product of cleavage could therefore be a terminal phosphate.

3 EXPERIMENTAL

3.1 General Procedures

Solvents and reagents

All solvents were of laboratory grade except those used for phosphoramidite extraction and purification when all solvents were of HPLC grade. Anhydrous dichloromethane, anhydrous triethylamine, anhydrous pyridine and anhydrous dioxane were prepared by distillation from calcium hydride. Doubly distilled pyridine was prepared by distillation from potassium hydroxide then calcium hydride. Anhydrous tetrahydrofuran was prepared by distillation from sodium/benzophenone. Anhydrous N,N-dimethylformamide was obtained by fractional distillation from molecular sieves. Anhydrous hexane and diethyl ether were dried over sodium wire, diisopropylamine was dried over calcium hydride. Anhydrous acetonitrile and 5% trichloroacetic acid in dichloromethane were purchased from Applied Biosystems Ltd.. 1M Triethylammonium bicarbonate solution was prepared by bubbling dry carbon dioxide through 34.7ml of triethylamine in 200ml water overnight then diluting with water to a 250ml volume.

4,4'-Dimethoxytrityl chloride was purchased from Courtaulds, 3-hydroxypropionitrile and 4-dimethylaminopyridine from Fluka, potassium phthalimide from Lancaster Synthesis, hydrazine hydrate and 2-nitrophenol from Fisons and 1-hydroxy-6-trifluoromethylbenzotriazole from Cambridge Research Biochemicals Ltd.. All other reagents were purchased from Aldrich. *p*-Toluenesulphonyl chloride was purchased from Aldrich and recrystallised from 40-60 petroleum ether/chloroform.

Chromatography

Flash column chromatography was carried out using silica-gel 60 supplied by Fluka. When the compound to be purified contained a component vulnerable to acid attack, such as phosphoramidite groups or 4,4'-dimethoxytrityl protected functionalities, the silica-gel was first washed with triethylamine in an appropriate co-

solvent. This neutralised all the acidic groups present on the silica-gel. Purification in the appropriate eluant could then proceed. For the phosphotriester monomers, in addition to priming with triethylamine, triethylamine was also added to the eluent during purification in order to maintain the monomers as their triethylammonium salts.

Thin layer chromatography (tlc) was carried out on aluminium sheets coated with silica 60, F_{254}, 0.2mm layer from Merck using the following solvent systems:

A Ethyl acetate:Diethyl ether (1:1)

B Hexane:Diethyl ether (1:1)

C Hexane:Ethyl acetate (1:1)

D Dichloromethane:Methanol (9:1)

E Toluene:Methanol (9:1)

F Ethyl acetate:Methanol:Ammonia (5:1:1)

Products were visualised on tlc using ultraviolet (UV) absorption at 264nm, phosphomolybdic acid (10%) in ethanol (dark blue colour in the presence of oxidisable compounds), an iodine tank (yellow stain in the presence of oxidisable compounds), ninhydrin spray (3% in ethanol, blue/black colour in the presence of amines) or Ellman's reagent [0.1% solution of dithio-bis-2-nitrobenzoic acid in ethanol/0.45M Tris buffer (1:1): bright yellow colour in the presence of thiols].

Nuclear Magnetic Resonance Spectra (NMR)

^{1}H-NMR spectra were recorded on a Brucker WH360 (360MHz), a Brucker WP200 (200.13MHz) or a Brucker WP80 (80MHz) spectrometer. ^{13}C-NMR spectra were recorded on a Brucker WH360 (90.6MHz) or a Brucker WP200 (50.32MHz) spectrometer. Trimethylsilane was used as internal reference and cited chemical shifts are given in ppm downfield to this standard. ^{31}P-NMR were recorded on a Brucker WP200 (81MHz) or a Jeol FX90Q (36MHz) spectrometer with phosphoric acid (δ 0.00) as an external reference. All samples were run in the solvent indicated.

Mass Spectra (MS)

Fast atom bombardment (FAB) mass spectra were recorded on a Kratos MS50TC mass spectrometer in a matrix of thioglycerol or 3-noba (3-nitrobenzylalcohol).

Infrared Spectra (IR)

Infrared spectra were recorded on a Biorad FTS-7 Fourier Transform IR spectrophotometer using KBr plates and a polystyrene reference ($1603cm^{-1}$). Samples were used undiluted or as a nujol mull.

CHN Microanalyses

CHN Microanalyses were carried out on a Perkin Elmer 240 elemental analyser.

Melting Points (MP)

Melting Points were carried out on a Kofler Hotstage apparatus and are uncorrected.

3.2 Oligonucleotide Synthesis, Analysis and Purification

Oligonucleotide Synthesis

Oligonucleotide syntheses were performed on Applied Biosystems 380B or 394 DNA synthesisers on a 0.2 or 1μmole scale. Standard cyanoethyl phosphoramidite monomers for DNA synthesis were supplied by Applied Biosystems Ltd., biotin monomers were supplied by Cambridge Research Biochemicals Ltd. and all monomers were used at a concentration of 0.1M in anhydrous acetonitrile. All other DNA synthesis reagents were supplied by Applied Biosystems Ltd.. Sephadex G25 NAP-10 (nucleic acid purification) columns were supplied by Pharmacia. For phosphorothioate oligonucleotide syntheses, iodine was replaced by tetraethylthiuram disulphide (TETD). Reverse osmosis purified sterile water was used at all stages of oligonucleotide synthesis, analysis and purification.

The overall yield of purified oligonucleotide in milligrams or micromoles can be determined from the UV absorbance of an aqueous solution of the oligonucleotide. Hence oligonucleotide quantity is often quoted in terms of optical density (OD) values.

Measurement of Coupling Efficiencies

Coupling efficiencies were measured by comparison of the absorbance at 495nm of the 4,4'-dimethoxytrityl cation produced in the detritylation steps of successive synthesis cycles. Absorbances were measured after diluting the individual trityl samples to 25ml with 0.1M *p*-toluenesulphonic acid in acetonitrile.

Coupling of the Lipophilic Monomers

All lipophilic monomers were used as 0.1M solutions in anhydrous dichloromethane. In order to increase the coupling yields of the lipophilic phosphoramidites, delivery times for the addition of these monomers were increased

to 4 seconds (from 2 seconds), coupling times were increased to 5 minutes (from 30 seconds) and oxidation times were increased to 15 minutes (from 30 seconds). In all cases an additional dichloromethane wash step before and after the coupling step was included in the synthesis cycle to prevent precipitation of the monomer and blockage of the reagent lines.

For the hexaethyleneglycol and hexadecyl monomers containing the 4,4'-dimethoxytriyl group, coupling efficiencies were measured directly on the DNA synthesiser via the Trityl-Off configuration. For the cholesterol, 1,2-di-O-hexadecylglyceryl and related monomers, coupling efficiencies were deduced by removing the capping step from the synthesis cycle of the lipophilic monomer addition, calculating the coupling efficiency of a further addition of a standard phosphoramidite monomer (e.g. dT) onto the unreacted 5'-hydroxyls of the oligonucleotide (assuming a standard coupling of 98.5% for the standard monomer).

Coupling of the Phosphotriester Monomers

All phosphotriester monomers were coupled onto a detritylated (5' Trityl-Off) support bound oligonucleotide by a non-automated procedure in the quantities indicated below (***Table 3.1***).

Table 3.1

Triester Compound	**41**	**42**	**43**	**46**	**54**	**57**
mass (mg)	108	94	115	104	140	109

The monomer (0.14mmol) and 1-(2-mesitylenesulphonyl)-1,2,4-triazole (MSNT, 3eq., 0.42mmol, 123mg) were dissolved in an anhydrous acetonitrile (anhydrous tetrahydrofuran for the cholesterol monomers)/doubly distilled pyridine mixture (9:1, 0.20ml) and were injected manually onto the DNA synthesis column. Following

intermittent agitation for 90 minutes, the column was washed repeatedly with anhydrous acetonitrile (or anhydrous tetrahydrofuran for the cholesterol monomers) then returned to the DNA synthesiser for all subsequent detritylations and couplings and for cleavage from the solid support.

Cleavage of the Disulphide Oligonucleotides

Following removal of the solvent under vacuum, the oligonucleotides were dissolved in 0.1ml of 0.1M ammonium acetate solution to which was added 0.2ml of aqueous ammonia and dithiothreitol (DTT, 50mg, 0.32mmol). The solution was left at room temperature for 1 hour then the oligonucleotide was purified by gel filtration using a Sephadex G25 NAP-10 column. Analysis and further purification were performed by HPLC as required.

Reaction of Thiol Oligonucleotide with 5-Iodoacetamidofluorescein[139]

Approximate 4.0OD units of the thiol oligonucleotide were dissolved in 0.3ml of 0.2M Tris/HCl buffer (pH 7.2-8). 5-Iodoacetamidofluorescein (3mg) was dissolved in anhydrous N,N-dimethylformamide (0.3ml) and added to the oligonucleotide. At this point the solution turned bright orange. After being left in the dark for 5 hours at room temperature, the mixture was purified twice by gel filtration using Sephadex G25 NAP-10 columns. Analysis was carried out on HPLC.

Derivatisation of Controlled Pore Glass (CPG) solid support

1. Activation

3-Aminopropyl CPG (0.5g) was slowly shaken in trichloroacetic acid (5% in dichloromethane, 5ml) for 2 hours at room temperature. The solution was filtered off and the glass beads washed successively with triethylamine/diisopropylethylamine (9:1), dichloromethane and diethyl ether. The resin was then dried under vacuum for 90 minutes.

2. Succinylation

The activated CPG (0.5g) was mixed with succinic anhydride (1mmol, 100mg) and 4-dimethylaminopyridine (0.33mmol, 40mg) in 4ml of anhydrous pyridine. After being slowly shaken for 18 hours at room temperature, the mixture was filtered and the glass beads washed successively with pyridine, dichloromethane and diethyl ether. The resin was then dried under vacuum for 90 minutes.

3. Functionalisation

A sample of the succinylated CPG (0.25g) was mixed with compound **[31]** (p50, 0.07mmol, 34mg), 4-dimethylaminopyridine (0.025mmol, 3mg) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.5mmol, 96mg) in 6ml of anhydrous pyridine. After being slowly shaken for 18 hours at room temperature, the mixture was filtered and the glass beads washed successively with pyridine, dichloromethane and diethyl ether. The resin was then dried under vacuum for 90 minutes.

4. Desuccinylation

To removed the unreacted succinylate groups, the functionalised CPG was slowly shaken with piperidine (5ml) for 5 minutes at room temperature. The glass beads were then filtered, washed successively with pyridine, dichloromethane and diethyl ether and dried under vacuum for 90 minutes.

5. Trityl Test

To measure the degree of functionalisation, the absorbance of the trityl colours was measured. Approximately 5mg of functionalised resin were suspended in trichloroacetic acid (5% in dichloromethane, 10ml) and the absorbance was measured at 504nm. The amount of bound monomer in $\mu mol.g^{-1}$ of support (c), is given by:

$$c = \frac{\text{absorbance at 504nm x volume (ml) of trichloroacetic acid solution x 13.2}}{\text{mass of resin (mg)}}$$

Absorbance measured = 2.38; mass = 5.4mg; therefore c = 58.0μmol.g^{-1}

6. Capping

To cap the unreacted amino functions, the CPG was mixed with N-methyl-imidazole (0.75ml) and acetic anhydride (1.65ml) in anhydrous tetrahydrofuran (4ml) and slowly shaken for 2 hours at room temperature. The glass beads were filtered then washed successively with pyridine, dichloromethane and diethyl ether. The resin was then dried under vacuum for 90 minutes.

This gave 184mg of functionalised CPG resin to be loaded into a synthesis column and used in automated DNA synthesis.

High Performance Liquid Chromatography (HPLC)

The fully deprotected oligonucleotides were analysed and purified by reverse phase HPLC on a Gilson 306 system using a Brownlee Aquapore Octyl (C8) reverse phase column (25cm x 10mm) with a flow rate of 3mlmin^{-1}. The following gradients were used:

Gradient **1**

Time (minutes)	0	2	20	27	28	30
% B	0	0	100	100	0	0

A = 0.1M triethylammonium acetate solution

B = 80% acetonitrile in 0.1M triethylammonium acetate solution

Gradient **2**

Time (minutes)	0	2	3	20	22	27	28	30
% B	0	0	15	50	100	100	0	0

A = 0.1M ammonium acetate solution

B = 50% acetonitrile in 0.1M ammonium acetate solution

Gradient **3**

Time (minutes)	0	2	3	24	25	27	28	30
% B	0	0	15	50	100	100	0	0

A = 0.1M ammonium acetate solution

B = 25% acetonitrile in 0.1M ammonium acetate solution

Oligonucleotide type	Lipophilic	Trityl-On/Biotin	Trityl-Off
Gradient	**1**	**2**	**3**

The gradients and acetonitrile concentrations used were reflective of the lipohilicity of the synthesised oligonucleotides. The UV monitor was set at a value between 264nm and 295nm depending on the length and quantity of the oligonucleotide being analysed. In most cases the desired oligonucleotides eluted several minutes after the failure sequences. If required the oligonucleotides were further purified by gel filtration using Sephadex G25 NAP-10 columns and then lyphophilised.

Capilliary Zone Gel Electrophoresis (CZE)

Further analysis of oligonucleotides by CZE were conducted on an Applied Biosystems Model 270A automated electrophoresis system with MicroGel capillaries. The following conditions were used:

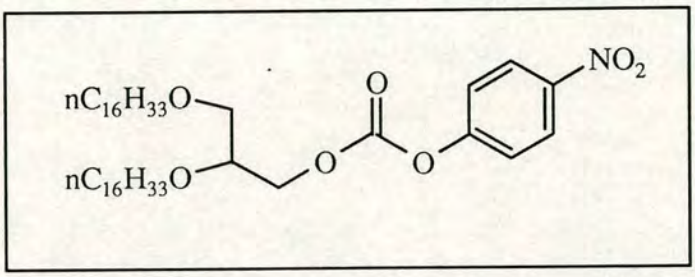
Buffer = 75mM Tris-phosphate/10% methanol, pH 7.5;

Loading voltage = -5kV; Temperature = 30^{O}C; Samples = 5μM in water;

Running voltage = -15kV; Detector = 260nm.

3.3 Synthetic Procedures

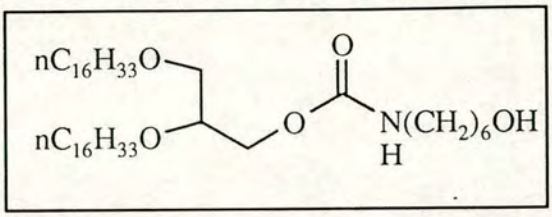
1,2-Di-O-hexadecylglyceroxy(*p*-nitrophenylcarbonate) [27]



To a stirred solution of bis(*p*-nitrophenyl)carbonate (2eq., 2mmol, 0.61g) in anhydrous dichloromethane (10ml), a solution of 1,2-di-O-hexadecylglycerol (1eq., 1mmol, 0.54g) in anhydrous dichloromethane (10ml) was added dropwise. 1,8-Diazabicyclo[5.4.0]undec-7-ene (2eq., 2mmol, 0.30ml) was then added. After stirring at room temperature for 18 hours, the mixture was then diluted with dichloromethane (30ml), washed with brine (3 x 30ml) then citric acid (3 x 30ml), at which point the organic layer became neutral. After drying over anhydrous sodium sulphate and removal of the solvent by evaporation, the product was purified by silica-gel flash chromatography, eluting with hexane:diethyl ether (1:1) to give a waxy, white solid.

Yield 1.00g (71%). R_f 0.87 (system B). MP 36-37°C. υ_{max}(film)/cm^{-1} 2958 (s), 2915 (s), 2848 (s), 1765 (s, CO), 1593 (w), 1524 (m), 1468 (m), 1349 (m), 1259 (s), 1217 (s). 1091 (s), 1018 (s), 861 (m), 798 (s). FAB mass spectrum: m/z 706.5572 [calc. for $C_{42}H_{76}NO_7$ (M^+ + 1): 706.5621]. ^{1}H-NMR ($CDCl_3$): δ 0.83-0.90 (t, 6H, $2CH_3$ C16. *J*=6.4Hz); 1.24 (s, 60H, $30CH_2$); 3.39-3.72 (m, 5H); 7.35-7.39 (d, 2H, 2, 6 CH nitrophenyl, $J_{2\text{-}3}$=9.2Hz); 8.24-8.29 (d, 2H, 5, 3 CH nitrophenyl, $J_{3\text{-}2}$=9.2Hz). ^{13}C-NMR ($CDCl_3$): δ 13.99 ($2CH_3$); 22.57 ($2CH_2$); 25.97 ($2CH_2$); 29.25 ($2CH_2$); 29.36 ($2CH_2$); 29.57 ($18CH_2$); 31.81 ($2CH_2$); 68.68 (CH_2O); 69.41 (CH_2O); 70.69 (CH_2O); 71.75 (CH_2O); 76.00 (CHO); 121.62 (2ArCH); 125.15 (2ArCH); 145.22 (ArC); 152.37 (ArC); 155.47 (CO). Analysis: 71.53% C, 10.85% H, 2.28% N (calc. for $C_{42}H_{75}NO_7$: 71.45% C, 10.71% H, 1.98% N).

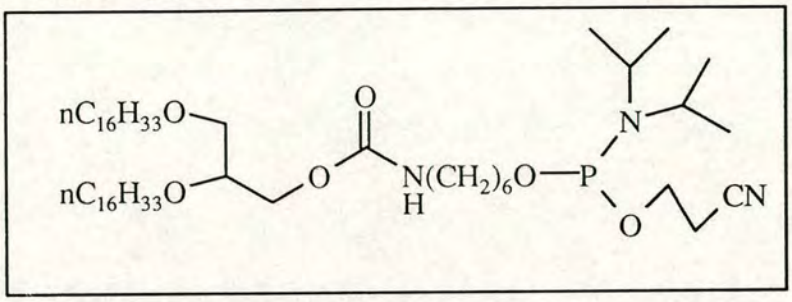
1-[(1,2-Di-O-hexadecylglyceroxy)carbonylamino]hexan-6-ol [28]



Compound **[27]** (1eq., 1.34mmol, 0.95g) was dissolved in anhydrous dichloromethane (6ml) to which was added 6-aminohexan-1-ol (1.1eq., 1.4mmol, 0.16g) and anhydrous triethylamine (1.1eq., 1.4mmol, 0.20ml). After stirring at room temperature for 19 hours, the solution was diluted with dichloromethane (30ml) then washed with brine (3 x 30ml) and water (30ml). After drying over anhydrous sodium sulphate and concentration by evaporation, the residue was purified by silica-gel flash chromatography, eluting with hexane:ethyl acetate (1:1) to give a waxy, white solid.

Yield 0.59g (64%). R_f 0.57 (system C). MP 49-50°C. υ_{max}(film)/cm^{-1} 3331 (s, OH), 2947 (s), 2918 (s), 2850 (s), 1686 (s, CO), 1534 (m), 1511 (s), 1376 (s), 1339 (w), 1286 (m), 1250 (s), 1218 (w), 1110 (m), 1062 (m), 1034 (w), 1012 (w), 720 (m). FAB mass spectrum: m/z 685 (M^+ + 1). ^{1}H-NMR ($CDCl_3$): δ 0.82-0.88 (t, 6H, $2CH_3$, C16, *J*=6.2Hz); 1.23 (s, 72H, $36CH_2$); 3.38-3.64 (m, 5H). ^{13}C-NMR ($CDCl_3$): δ 13.99 ($2CH_3$); 22.56 ($2CH_2$); 25.16 (CH_2); 25.95 ($2CH_2$); 26.23 ($2CH_2$); 29.24 ($2CH_2$); 29.57 ($18CH_2$); 29.86 ($2CH_2$); 31.80 ($2CH_2$); 32.39 (CH_2); 40.72 (CH_2N); 62.51 (CH_2O); 64.07 (CH_2O); 70.23 (CH_2O); 70.46 (CH_2O); 71.66 (CH_2O); 76.71 (CHO); 156.42 (CO). Analysis: 73.45% C, 12.44% H, 2.46% N (calc. for $C_{42}H_{85}NO_5$: 73.74% C, 12.52% H, 2.05% N).

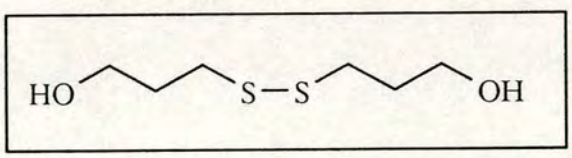
2-Cyanoethyl-N,N-diisopropyl{1-[(1,2-di-O-hexadecylglyceroxy)carbonylamino]hexan-6-O}phosphoramidite [29]



Compound **[28]** (1eq., 0.51g, 0.75mmol) was coevaporated twice with anhydrous dichloromethane then dissolved in anhydrous dichloromethane (8ml). Diisopropylethylamine (3eq., 2.25mmol, 0.29g, 0.39ml) and 2-cyanoethyl-N,N-diisopropylphosphoramidite[163] (1.4eq., 1.05mmol, 0.25g, 0.24ml) were added. After stirring at room temperature for 24 hours, the reaction mixture was diluted with ethyl acetate, washed with brine then dried (anhydrous sodium sulphate) and concentrated under vacuum. The product was purified by silica-gel flash chromatography, eluting with anhydrous hexane:anhydrous diethyl ether (1:1) primed with 1% triethylamine.

Yield 0.28g (42%). R_f 0.43 (system B). FAB mass spectrum: m/z 886 (M^+ + 3). ^{1}H-NMR ($CDCl_3$): δ 0.82-0.89 (t, 6H, $2CH_3$ C16, *J*=6.4Hz); 1.13-1.15 (d, 6H, $2CH_3$ i-Pr, *J*=2.3Hz); 1.17-1.18 (d, 6H, $2CH_3$ i-Pr, *J*=2.3Hz); 1.23 (s, 72H, $36CH_2$); 2.59-2.65 (m, 2H, CH_2CN); 3.37-3.64 (m, 7H); 3.77-3.84 (m, 2H, CH_2O). ^{31}P-NMR ($CDCl_3$): δ 147.67 (s).

Dithiodipropan-1,1'-ol [30]

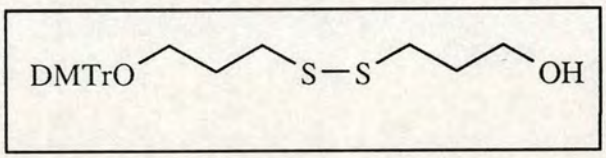


3-Bromopropan-1-ol (1eq., 72mmol, 10g) and sodium thiosulphate (1eq., 72mmol, 17.9g) were added to an ethanol/water mixture (1:1, 100ml) and refluxed for 30 minutes to allow a homogeneous solution to form. After cooling, iodine (1eq.,

72mmol, 9.1g) was added in small portions, at which point the violet colour remained. The reaction mixture was diluted with diethyl ether (50ml) and 0.1M potassium chloride solution (50ml). The product was extracted into the organic layer with diethyl ether (4 x 50ml) and the combined extracts washed with 1M sodium sulphite solution (3 x 50ml) until colourless. After drying (anhydrous sodium sulphate) and removal of the solvent under vacuum, the product, a clear oil, did not require any further purification.

Yield 5.35g (82%). R_f 0.46 (system D). υ_{max}(film)/cm^{-1} 3501 (s, OH), 3261 (s, OH), 2930 (s), 2876 (s), 1642 (s), 1470 (m), 1433 (s), 1346 (m), 1283 (m), 1257 (s), 1148 (m), 1052 (s), 901 (s). FAB mass spectrum: m/z 182.0423 [calc. for $C_6H_{14}O_2S_2$ (M^+): 182.0435]. ^{1}H-NMR ($CDCl_3$): δ 1.85-1.98 (quin., 4H, 2, 2' CH_2, *J*=6.6Hz); 2.74-2.81 (t, 4H, 3, 3' CH_2, *J*=7.1Hz); 3.68-3.74 (t, 4H, 1, 1' CH_2, *J*=6.1Hz). ^{13}C-NMR ($CDCl_3$): δ 31.59 ($2CH_2$); 34.97 ($2CH_2S$); 60.62 ($2CH_2O$).

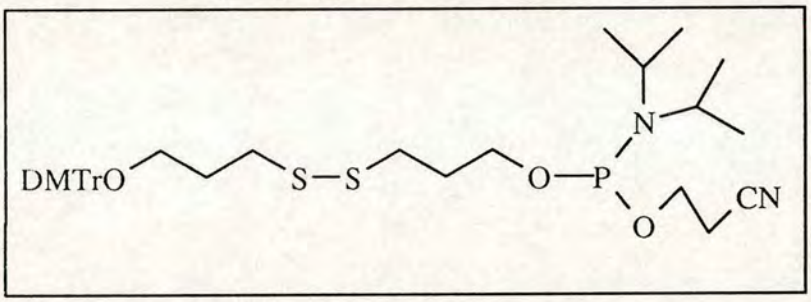
1-(4,4'-Dimethoxytrityloxy)dithiodipropan-1'-ol [31]



Compound **[30]** (1eq., 3.08g, 16.9mmol) was dried twice using anhydrous pyridine then dissolved in anhydrous pyridine (15ml) to which was added 4,4'-dimethoxytrityl chloride (1eq., 16.9mmol, 5.73g). After stirring at room temperature for 16 hours the reaction mixture was quenched with methanol (30ml) then evaporated to an oil which was coevaporated twice with toluene. The residue was taken up in dichloromethane (50ml), washed with brine (3 x 30ml) then dried (anhydrous sodium sulphate). The solvent was removed by evaporation and the product was purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-4%) primed with 1% triethylamine.

Yield 4.51g (55%). R_f 0.36 (system E). υ_{max}(film)/cm^{-1} 3409 (s, OH), 3055 (w), 2951 (m), 2928 (s), 2835 (w), 1607 (s), 1580 (w), 1462 (m), 1443 (m), 1299 (m), 1249 (s), 1176 (s), 1153 (w), 1066 (m), 1034 (s), 901 (w), 828 (s), 753 (m), 702 (m). FAB mass spectrum: m/z 485.1787 [calc. for $C_{27}H_{33}O_4S_2$ (M^+ + 1): 485.1820]. ^{1}H-NMR ($CDCl_3$): δ 1.90-1.97 (m, 4H, 2, 2' CH_2); 2.71-2.84 (m, 4H, 3, 3' CH_2); 3.12-3.18 (t, 2H, 1 CH_2, *J*=6.1Hz); 3.68-3.74 (t, 2H, 1' CH_2, *J*=6.1Hz); 3.78 (s, 6H, $2OCH_3$); 6.79-6.85, 7.19-7.44 (m, 13H, ArH). ^{13}C-NMR ($CDCl_3$): δ 29.58 (CH_2); 31.70 (CH_2); 34.99 (CH_2S); 35.61 (CH_2S); 55.00 ($2OCH_3$); 60.58 (CH_2O); 61.46 (CH_2O); 85.63 (C); 112.81 (4ArCH); 126.47 (ArCH); 127.56 (2ArCH); 127.91 (2ArCH); 129.80 (4ArCH); 136.17 (2ArC); 144.95 (ArC); 158.13 (2ArC).

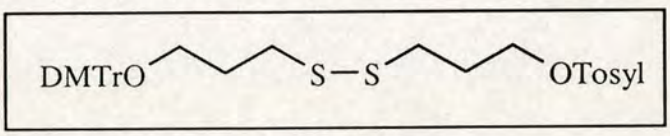
2-Cyanoethyl-N,N-diisopropyl[1-(4,4'-dimethoxytrityloxy)dithiodipropan-1'-O]phosphoramidite [32]



Compound **[31]** (1eq., 0.96g, 1.98mmol) was coevaporated twice with anhydrous dichloromethane then dissolved in anhydrous dichloromethane (10ml). Diisopropylethylamine (3eq., 5.94mmol, 0.77g, 1.03ml) and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite[163] (1eq., 1.98mmol, 0.47g, 0.44mmol) were added dropwise. After stirring at room temperature for 20 minutes, the reaction mixture was quenched with ethyl acetate, washed quickly with brine, dried (anhydrous sodium sulphate) and concentrated under vacuum. The product was purified by silica-gel flash chromatography, eluting with anhydrous hexane:anhydrous diethyl ether (1:1) primed with 1% triethylamine.

Yield 0.46g (34%). R_f 0.42 (system B). FAB mass spectrum: m/z 684 (M^+). ^{1}H-NMR ($CDCl_3$): δ 1.14-1.42 (m, 12H, $4CH_3$ i-Pr); 1.88-2.11 (m, 4H, 2, 2' CH_2); 2.67-2.80 (m, 6H, CH_2CN, 3, 3' CH_2); 2.94-3.01 (m, 2H, 2CH i-Pr); 3.13-3.19 (t, 2H, 1 CH_2, *J*=5.9Hz); 3.45-3.59 (m, 2H, CH_2O); 3.76-3.82 (m, 8H, 1 CH_2, $2OCH_3$); 6.78-6.84, 7.15-7.45 (m, 13H, ArH). ^{31}P-NMR ($CDCl_3$): δ 149.69 (s).

1-(4,4'-Dimethoxytrityloxy)dithiodipropan-1'-O-(*p*-toluenesulphonate) [33]

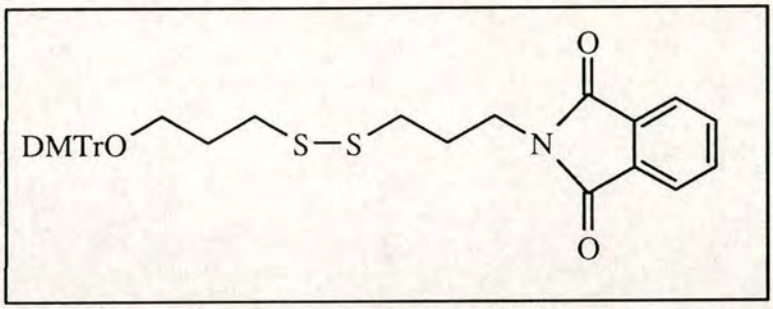


To a solution of compound **[31]** (1eq., 9.57mmol, 4.64g) in anhydrous pyridine (60ml) cooled to 0°C was added *p*-toluenesulphonyl chloride (1.2eq., 11.48mmol, 2.19g). After stirring for 30 minutes at 0°C and a further 16 hours at 20°C, the solution was evaporated to an oil which was coevaporated twice with toluene. The residue was taken up in dichloromethane (50ml) and washed successively with water (30ml) and brine (3 x 30ml). The organic layer was dried (anhydrous sodium sulphate) and the solvent removed under vacuum. The product was purified by silica-gel flash chromatography eluting with methanol in dichloromethane (0-2%) primed with 1% triethylamine.

Yield 2.88g (47%). R_f 0.78 (system E). υ_{max}(film)/cm^{-1} 3056 (m), 2930 (s), 2835 (m), 1606 (s), 1581 (m), 1508 (s), 1361 (s), 1299 (m), 1249 (s), 1176 (s), 1033 (s), 986 (m), 919 (s), 827 (s), 736 (s), 663 (s). FAB mass spectrum: m/z 638.1851 [calc. for $C_{31}H_{38}O_6S_2$ (M^+): 638.1830]. ^{1}H-NMR ($CDCl_3$): δ 1.87-2.07 (m, 4H, 2, 2' CH_2); 2.43 (s, 3H, Ar-CH_3); 2.58-2.78 (m, 4H, 3, 3' CH_2); 3.10-3.16 (t, 2H, 1 CH_2, *J*=6.1Hz); 3.68-3.75 (t, 2H, 1' CH_2, *J*=6.1Hz); 3.78 (s, 6H, $2OCH_3$); 6.78-6.84, 7.13-7.44, 7.75-7.80 (m, 17H, ArH). ^{13}C-NMR ($CDCl_3$): δ 21.51 (ArCH_3); 27.98 (CH_2); 29.54 (CH_2); 33.66 (CH_2S); 35.49 (CH_2S); 55.05 ($2OCH_3$); 60.73 (CH_2O); 61.37 (CH_2O); 85.66 (C); 112.84 (2ArCH); 112.94 (2ArCH); 126.52 (ArCH); 127.59

(2ArCH); 127.73 (2ArCH); 127.93 (2ArCH); 128.95 (2ArCH); 129.79 (4ArCH); 132.57 (ArC); 136.14 (2ArC); 139.27 (ArC); 144.93 (ArC); 158.18 (2ArC).

N-Phthaloyl-1'-amino-1-(4,4'-dimethoxytrityloxy)dithiodipropane [34]

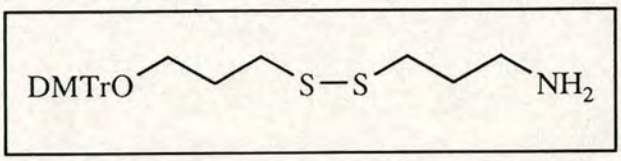


To a solution of compound **[33]** (1eq., 1.36mmol, 0.86g) in anhydrous N,N-dimethylformamide (20ml) was added potassium phthalimide (1.1eq., 1.56mmol, 0.29g). The reaction mixture was stirred for 2 hours at 55°C at which point it was diluted with dichloromethane (20ml) and washed with water (3 x 30ml). The organic layer was dried (anhydrous sodium sulphate) and concentrated under vacuum. The residue was purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-2%) primed with 1% triethylamine.

Yield 0.74g (86%). R_f 0.67 (system E). υ_{max}(film)/cm^{-1} 3055 (m), 2930 (s), 2835 (m), 1771 (m, CO), 1713 (s, CO), 1676 (s, CO), 1607 (s), 1580 (w), 1508 (s), 1394 (s), 1365 (w), 1300 (m), 1249 (s), 1176 (s), 1154 (w), 1086 (s), 1033 (s), 1011 (w), 902 (w), 829 (s), 754 (w), 721 (s), 658 (w). FAB mass spectrum: m/z 613.1969 [calc. for $C_{35}H_{35}NO_5S_2$ (M^+): 613.1957]. ^{1}H-NMR ($CDCl_3$): δ 1.91-2.11 (m, 4H, 2, 2' CH_2); 2.62-2.70 (t, 2H, 3 CH_2, *J*=7.1Hz); 2.75-2.82 (t, 2H, 3' CH_2, *J*=7.1Hz); 3.10-3.16 (t, 2H, 1 CH_2, *J*=6.1Hz); 3.72-3.78 (m, 8H, 2OCH_3, 1' CH_2); 6.77-6.82, 7.16-7.43, 7.65-7.83 (m, 17H, ArH). ^{13}C-NMR ($CDCl_3$): δ 27.94 (CH_2); 29.60 (CH_2); 35.64 (CH_2S); 35.73 (CH_2S); 36.58 (CH_2N); 54.98 (2OCH_3); 61.43 (CH_2O); 85.61 (C); 112.79 (4ArCH); 123.05 (2ArCH); 126.44 (ArCH); 127.53

(2ArCH); 127.92 (2ArCH); 129.78 (4ArCH); 131.80 (2ArC); 133.79 (2ArCH); 136.17 (2ArC); 144.94 (ArC); 158.12 (2ArC); 168.10 (2CO).

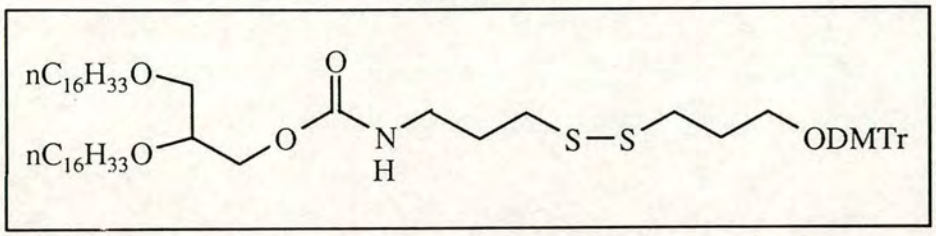
1'-Amino-1-(4,4'-dimethoxytrityloxy)dithiodipropane [35]



To a solution of compound **[34]** (1eq., 3.21mmol, 1.97g) in dichloromethane/methanol (1:1, 15ml) was added hydrazine hydrate (1.1eq., 3.53mmol, 0.18g, 0.17ml). The mixture was stirred for 22 hours at room temperature then concentrated under vacuum. The residue was dissolved in dichloromethane (40ml) and washed with 2M sodium hydroxide solution (30ml). The aqueous phase was extracted with diethyl ether (3 x 30ml) and the combined organic layers were dried (anhydrous sodium sulphate) and concentrated under vacuum. The residue was purified by silica-gel flash chromatography, eluting with dichloromethane:methanol (9:1) primed with 1% triethylamine.

Yield 0.87g (56%). R_f 0.18 (system D). υ_{max}(film)/cm^{-1} 3412 (m, NH), 3374 (m, NH), 2929 (s), 2834 (m), 1607 (s), 1580 (w), 1508 (s), 1299 (m), 1249 (s), 1175 (s), 1033 (s), 827 (s). FAB mass spectrum: m/z 485.2069 [calc. for $C_{27}H_{35}NO_3S_2$ (M^+ + 2): 485.2058]. ^{1}H-NMR ($CDCl_3$): δ 1.74-1.82 (t, 2H, 2 CH_2, *J*=6.6Hz); 1.93-2.00 (t, 2H, 2' CH_2, *J*=6.6Hz); 2.66-2.83 (m, 6H, 1', 3, 3' CH_2); 3.11-3.17 (t, 2H, 1 CH_2, *J*=6.1Hz); 3.77 (s, 6H, 2OCH_3); 6.78-6.83, 7.14-7.43 (m, 13H, ArH). ^{13}C-NMR ($CDCl_3$): δ 29.62 (CH_2); 32.71 (CH_2); 35.67 (CH_2S); 36.04 (CH_2S); 40.55 (CH_2N); 55.01 (2OCH_3); 61.49 (CH_2O); 85.64 (C); 112.81 (4ArCH); 126.48 (ArCH); 127.57 (2ArCH); 127.93 (2ArCH); 129.81 (4ArCH); 136.18 (2ArC); 144.96 (ArC); 158.16 (2ArC).

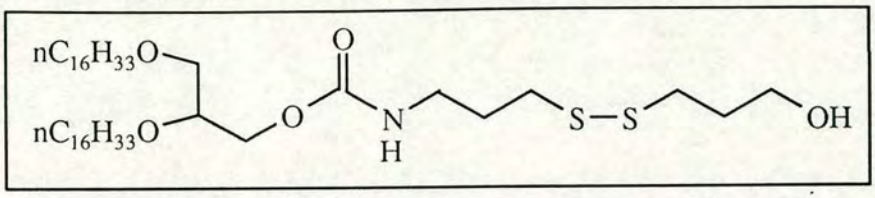
1'-[(1,2-Di-O-hexadecylglyceroxy)carbonylamino]-1-(4,4'-dimethoxytrityloxy)dithiodipropane [36]



Compound **[35]** (1eq., 0.23g, 0.47mmol) was coevaporated twice with anhydrous dichloromethane then dissolved in anhydrous dichloromethane (10ml) to which was added compound **[27]** (1eq., 0.47mmol, 0.33g) and anhydrous triethylamine (1eq., 0.47mmol, 0.048g, 0.066ml). After stirring at room temperature for 20 hours, dichloromethane was added (20ml) and the resulting solution washed with 0.5M sodium hydrogen carbonate solution (20ml), brine (3 x 20ml) and water (20ml). The organic layer was dried (anhydrous sodium sulphate), concentrated under vacuum, then the product was purified by silica-gel flash chromatography, eluting with hexane:ethyl acetate (1:1) primed with 1% triethylamine.

Yield 0.41g (82%). R_f 0.53 (system B). υ_{max}(film)/cm^{-1} 3391 (w, NH), 2922 (s), 2851 (s), 1740 (s, CO), 1607 (s), 1508 (s), 1463 (s), 1372 (s), 1299 (w), 1245 (s), 1175 (m), 1114 (m), 1043 (s), 827 (m), 791 (w). FAB mass spectrum: m/z 1049.7242 [calc. for $C_{63}H_{103}NO_7S_2$ (M^+): 1049.7176]. ^{1}H-NMR ($CDCl_3$): δ 0.84-0.87 (t, 6H, $2CH_3$ C16, J=6.5Hz); 1.25 (s, 60H, $30CH_2$); 1.86-1.96 (m, 4H, 2, 2' CH_2); 2.61-2.79 (m, 4H, 3, 3' CH_2); 3.15 (m, 2H, 1 CH_2); 3.39-3.54 (m, 7H, 1' CH_2, 5H C35); 3.79 (s, 6H, $2OCH_3$); 6.80-7.00, 7.25-7.43 (m, 13H, ArH).

1'-[(1,2-Di-O-hexadecylglyceroxy)carbonylamino]dithiodipropan-1-ol [37]

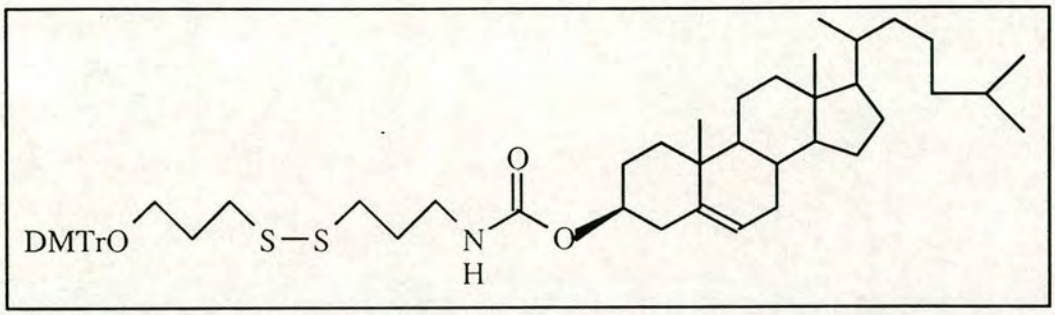


Compound **[36]** (0.34g, 0.32mmol) was dissolved in trichloroacetic acid (5% in dichloromethane, 5ml) and stirred at room temperature for 16 hours. The reaction mixture was diluted with dichloromethane (20ml), washed with 0.5M sodium hydrogen carbonate solution until the organic layer became neutral (x5) and dried (anhydrous sodium sulphate). The solvent was removed under vacuum and the product was purified by silica-gel flash chromatography, eluting with hexane:ethyl acetate (1:1) to give a white solid.

Yield 0.090g (38%). R_f 0.50 (system C). MP 45-47^{o}C. υ_{max}(film)/cm^{-1} 3315 (s, OH), 3013 (w), 2959 (s), 2917 (s), 2849 (s), 1680 (s, CO), 1607 (m), 1540 (m), 1468 (s), 1454 (s), 1375 (m), 1260 (s), 1095 (s), 1017 (s), 875 (w), 805 (s). FAB mass spectrum: m/z 748.59473 [calc. for $C_{42}H_{86}NO_5S_2$ (M^+ + 1): 748.59471]. ^{1}H-NMR ($CDCl_3$): δ 0.85-0.88 (t, 6H, $2CH_3$ C16, *J*=6.7Hz); 1.24 (s, 60H, $30CH_2$); 1.86-1.98 (m, 4H, 2, 2' CH_2); 2.69-2.73 (t, 2H, 3' CH_2, *J*=7.1Hz); 2.79-2.83 (t, 2H, 3 CH_2, *J*=7.1Hz); 3.25-3.47 (m, 7H, 1' CH_2, 5H C35); 3.73-3.76 (t, 2H, 1 CH_2, *J*=6.0Hz). Analysis: 67.75% C, 11.63% H, 2.06% N (calc. for $C_{42}H_{85}NO_5S_2$: 67.42% C, 11.45% H, 1.87% N).

1'-(Cholesteryl-3-carbonylamino)-1-(4,4'-dimethoxytrityloxy)dithiodipropane

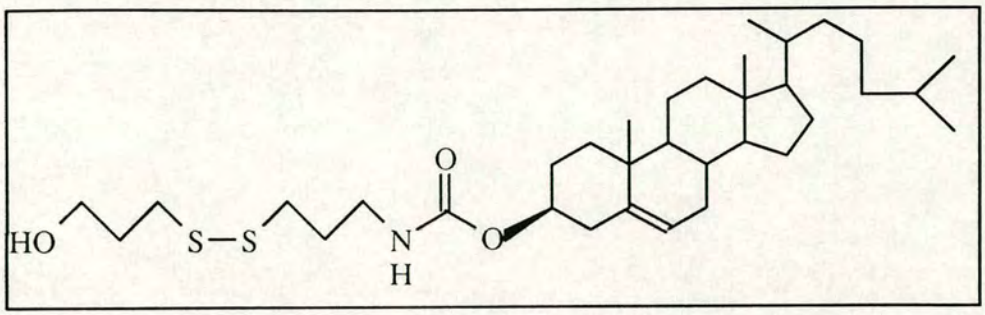
[38]



Compound **[35]** (1eq., 1.03mmol, 0.50g) was coevaporated twice with anhydrous dichloromethane then dissolved in anhydrous dichloromethane (10ml). To this was added cholesteryl chloroformate **[1]** (1eq., 1.03mmol, 0.46g) and anhydrous triethylamine (1eq., 1.03mml, 0.10g, 0.14ml). After stirring at room temperature for 24 hours, the reaction mixture was diluted with dichloromethane (20ml) then washed with 0.5M sodium hydrogen carbonate solution (20ml), brine (3 x 20ml) and water (20ml). The organic layer was dried (anhydrous sodium sulphate), the solvent removed under vacuum and the product purified by silica-gel flash chromatography, eluting with hexane:diethyl ether (1:1) primed with 1% triethylamine.

Yield 0.80g (87%). R_f 0.34 (system B). υ_{max}(film)/cm^{-1} 3365 (w, NH), 2952 (s), 2926 (s), 2852 (s), 1703 (s, CO), 1607 (s), 1508 (s), 1463 (s), 1378 (m), 1299 (m), 1249 (s), 1176 (s), 1082 (m), 1037 (s), 1012 (m), 902 (w), 828 (s), 701 (m). FAB mass spectrum: m/z 895.52426 [calc. for $C_{55}H_{77}O_5NS_2$ (M^+): 895.52428]. ^{1}H-NMR ($CDCl_3$): δ 0.67-1.60 (m, 45H, all signals cholesterol moiety); 1.82-2.03 (m, 4H, 2, 2' CH_2); 2.62-2.69 (t, 2H, 3' CH_2, *J*=7.1Hz); 2.76-2.83 (t, 2H, 3 CH_2, *J*=7.1Hz); 3.12-3.26 (m, 4H, 1, 1' CH_2); 3.79 (s, 6H, 2OCH_3); 6.80-6.84, 7.15-7.45 (m, 13H, ArH).

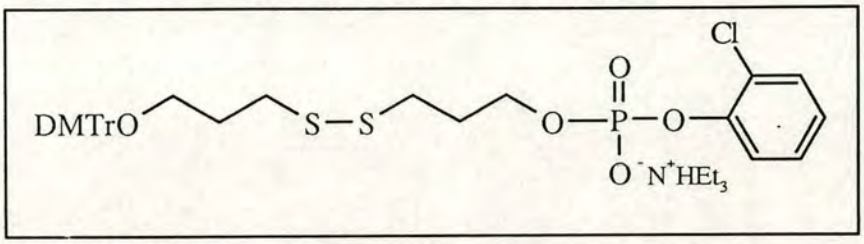
1'-(Cholesteryl-3-carbonylamino)dithiodipropan-1-ol [39]



Compound **[38]** (0.74g, 0.83mmol) was dissolved in trichloroacetic acid (5% in dichloromethane, 10ml) and stirred at room temperature for 16 hours. The reaction mixture was diluted with dichloromethane (20ml), washed with sodium hydrogen carbonate solution until the organic layer became neutral (x5) then dried over anhydrous sodium sulphate. After the solvent was removed under vacuum, the product required no further purification.

Yield 0.39g (79%). R_f 0.44 (system C). υ_{max}(film)/cm^{-1} 3349 (s, OH), 2929 (s), 2849 (s), 1693 (s, CO), 1530 (s), 1466 (s), 1439 (s), 1252 (s), 1134 (m), 1044 (s), 1015 (m), 840 (w). FAB mass spectrum: m/z 594.40143 [calc. for $C_{34}H_{60}NO_3S_2$ (M^+ + 1): 594.40144]. ^{1}H-NMR ($CDCl_3$): δ 0.67-1.59 (m, 45H, all signals cholesterol moiety); 1.76-1.98 (m, 4H, 2, 2' CH_2); 2.69-2.72 (t, 2H, 3' CH_2, *J*=7.1Hz); 2.78-2.81 (t, 2H, 3 CH_2, *J*=7.1Hz); 3.21-3.28 (m, 2H, 1' CH_2); 3.61-3.70 (m, 2H, 1, CH_2).

Triethylammonium[1-(4,4'-dimethoxytrityloxy)dithiodipropan-1'-O-(2-chlorophenylphosphate)] [41]

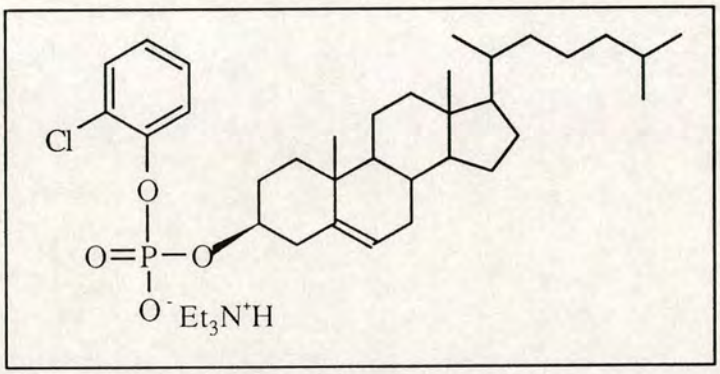


Compound **[31]** (1eq., 0.98g, 2.02mmol) was coevaporated twice with anhydrous pyridine then dissolved in anhydrous tetrahydrofuran (30ml). This was added to a solution of 2-chlorophenyldichlorophosphate (1.5eq., 3.03mmol, 0.74g, 0.49ml) and anhydrous triethylamine (3eq., 6.06mmol, 0.61g, 0.84ml) in anhydrous tetrahydrofuran (30ml). After stirring for 2 hours at room temperature, the reaction was quenched with aqueous 1.0M triethylammonium bicarbonate (30ml) and left stirring for a further 2 hours. Dichloromethane (30ml) was added and the organic layer was washed again with aqueous 1.0M triethylammonium bicarbonate (2 x 30ml), water (30ml) then dried (anhydrous sodium sulphate). The solvent was removed by evaporation and the product purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-5%) + 1% triethylamine to give a yellow oil.

Yield 1.34g (85%). R_f 0.37 (system F). υ_{max}(film)/cm^{-1} 3451 (s, OH), 3392 (s, OH), 2953 (s), 2927 (s), 2835 (s), 2674 (s), 2489 (s), 1607 (s), 1585 (m), 1505 (s), 1444 (m), 1392 (w), 1298 (m), 1247 (s), 1176 (s), 1092 (s), 1032 (s), 949 (m), 903 (s), 828 (s), 756 (s). FAB mass spectrum: m/z 675.14028 [calc. for $C_{33}H_{33}ClO_7PS_2$ (M^+): 675.14069]. ^{1}H-NMR ($CDCl_3$): δ 1.09-1.16 (t, 9H, $3CH_3$ Eth, J=7.3Hz); 1.87-1.97 (m, 4H, 2, 2' CH_2); 2.63-2.80 (m, 10H, 3, 3' CH_2, $3CH_2$ Eth); 3.07-3.13 (t, 2H, 1 CH_2, J=6.0Hz); 3.73 (s, 6H, $2OCH_3$); 4.02-4.08 (m, 2H, 1' CH_2); 6.75-7.42 (m, 16H, ArH); 7.61-7.66 (dd, 1H, 5 CH chlorophenyl, $J_{5\text{-}6}$=1.3Hz, $J_{4\text{-}5}$=8.2Hz). ^{13}C-NMR ($CDCl_3$): δ 9.70 ($3CH_3$); 29.55 (CH_2); 30.09 (CH_2); 34.79 (CH_2S); 35.54 (CH_2S); 45.61 ($3CH_2N$); 54.97 ($2CH_3$); 61.46 (CH_2O); 64.37 (CH_2O); 85.58 (C);

112.77 (4ArCH); 121.10 (ArCH); 123.16 (ArCH); 124.69 (ArC); 126.41 (ArCH); 127.29 (ArCH); 127.50 (2ArCH); 127.89 (2ArCH); 129.60 (ArCH); 129.75 (4ArCH); 136.16 (2ArC); 144.92 (ArC); 149.22 (ArC); 158.10 (2ArC). ^{31}P-NMR ($CDCl_3$): δ -4.49 (s).

Triethylammonium[cholesteryl-3-(2-chlorophenylphosphate)] [42]

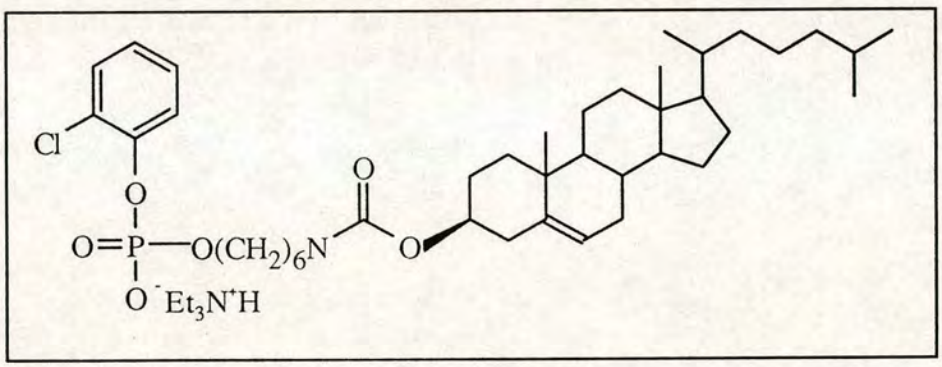


Cholesterol (1eq., 0.39g, 1.00mmol) was coevaporated with anhydrous pyridine then dissolved in anhydrous tetrahydrofuran (15ml). This was added to a solution of 2-chlorophenyldichlorophosphate (1.5eq., 1.50mmol, 0.37g, 0.24ml) and anhydrous triethylamine (3eq., 3.00mmol, 0.30g, 0.42ml) in anhydrous tetrahydrofuran (15ml). After stirring at room temperature for 24 hours, the reaction was quenched with aqueous 1.0M triethylammonium bicarbonate (20ml) and allowed to stir for a further 2 hours. Dichloromethane (20ml) was added and the organic layer was washed again with aqueous 1.0M triethylammonium bicarbonate (20ml) and water (20ml). After drying over anhydrous sodium sulphate and removal of the solvent under vacuum, the product was purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-5%) + 1% triethylamine to give a white solid.

Yield 0.46g (68%). R_f 0.42 (system F). MP 215-218^{o}C. υ_{max}(nujol)/cm^{-1} 3451 (s, OH), 2954 (s), 2923 (s), 2853 (s), 2620 (m), 2369 (m), 1585 (s), 1479 (s), 1280 (m), 1244 (s), 1094 (s), 1033 (s), 1024 (m), 899 (s), 888 (s), 846 (w), 767 (m), 739 (m), 677 (m). FAB mass spectrum: m/z 575 (M^+). ^{1}H-NMR ($CDCl_3$): δ 0.61-

1.57 (m, 54H, $3CH_3$ Eth, all other signals cholesterol moiety); 2.89-3.00 (q, 6H, $3CH_2$ Eth, J=7.3Hz); 6.83-7.70 (m, 4H, ArH). ^{13}C-NMR ($CDCl_3$): δ 8.52 ($3CH_3$); 11.63 (CH_3); 18.49 (CH_3); 19.14 (CH_3); 20.81 (CH_2); 22.36 (CH_3); 23.61 (CH_2); 24.07 (CH_2); 27.78 (CH); 28.03 (CH_2) 29.61 (CH_2); 31.65 (CH_2, CH); 35.58 (CH); 35.95 (CH_2); 36.23 (C); 36.92 (CH_2); 39.28 (CH_2); 39.52 (CH_2); 39.98 (CH_2); 42.06 (C); 45.29 ($3CH_2N$); 49.78 (CH); 55.90 (CH); 56.48 (CH); 75.83 (CH); 121.09 (ArCH); 121.56 (CH); 122.77 (ArCH); 124.42 (ArC); 127.22 (ArCH); 129.46 (ArCH); 140.39 (C); 149.46 (ArC). ^{31}P-NMR ($CDCl_3$): δ -5.12 (s). Analysis: 69.22% C, 9.50% H, 2.11% N (calc. for $C_{39}H_{65}ClNO_4P$: 69.05% C, 9.66% H, 2.06% N).

Triethylammonium[1-(cholesteryl-3-carbonylamino)hexan-6-O-(2-chlorophenylphosphate)] [43]

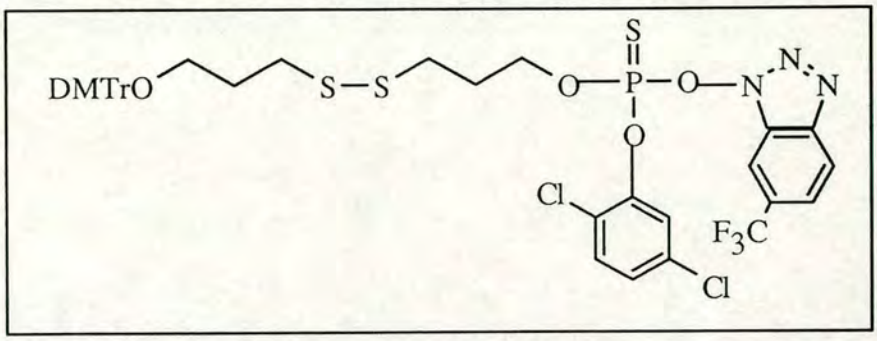


1-(Cholesteryl-3-carbonylamino)hexan-6-ol[104] (1eq., 1.65g, 3.11mmol) was coevaporated twice with anhydrous pyridine then dissolved in anhydrous tetrahydrofuran (30ml) to which was added 2-chlorophenyldichlorophosphate (1.5eq., 4.67mmol, 1.14g, 0.76ml) and anhydrous triethylamine (3eq., 9.33mmol, 0.94g, 1.30ml). After stirring for 5 hours at room temperature, the reaction was quenched with aqueous 1.0M triethylammonium bicarbonate (20ml) and left stirring for another 2 hours. Dichloromethane (30ml) was added and the organic layer was washed again with aqueous 1.0M triethylammonium bicarbonate (30ml) then water (30ml). The

solution was dried (anhydrous sodium sulphate), the solvent removed by evaporation and the product purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-5%) + 1% triethylamine to give a white solid.

Yield 1.45g (57%). R_f 0.34 (system F). MP 198-200°C. υ_{max}(nujol)/cm^{-1} 3362 (m), 2951 (s), 2922 (s), 2851 (s), 2675 (m), 2534 (m), 2337 (m), 1711 (s, CO), 1586 (m), 1242 (s), 1091 (s), 1016 (m), 905 (s), 672 (w). FAB mass spectrum: m/z 720 (M^+). ^{1}H-NMR ($CDCl_3$): δ 0.61-1.58 (m, 62H, $3CH_3$ Eth, $4CH_2$, all other signals cholesterol moiety); 2.93-3.04 (m, 8H, $3CH_2$ Eth, CH_2N); 3.88-3.97 (m, 2H, CH_2O); 6.84-7.29 (m, 3H, ArH); 7.59-7.64 (dd, 1H, 5 CH chlorophenyl, J_{5-6}=1.2Hz, J_{4-5}=8.2Hz). ^{13}C-NMR ($CDCl_3$): δ 8.28 ($3CH_3$); 11.60 (CH_3); 18.46 (CH_3); 19.09 (CH_3); 20.77 (CH_2); 22.32 (CH_3); 22.59 (CH_3); 23.56 (CH_2); 24.02 (CH_2); 25.07 (CH_2); 26.06 (CH_2); 27.74 (CH); 27.96 (CH_2); 29.64 (CH_2); 30.12 (CH_2); 30.27 (CH_2); 31.61 (CH, CH_2); 35.53 (CH); 35.91 (CH_2); 36.28 (C); 36.74 (CH_2); 38.33 (CH_2); 39.25 (CH_2); 39.46 (CH); 40.50 (CH_2); 42.03 (C); 45.26 ($3CH_2N$); 49.72 (CH); 55.84 (CH); 56.41 (CH); 65.85 (CH_2); 73.77 (CH); 121.06 (ArCH); 122.09 (CH); 122.95 (ArCH); 124.47 (ArC); 127.20 (ArCH); 129.50 (ArCH); 139.65 (C); 149.25 (ArC); 155.93 (CO). ^{31}P-NMR ($CDCl_3$): δ -4.60 (s). Analysis: 67.59% C, 9.48% H, 3.30% N (calc. for $C_{46}H_{78}ClN_2O_6P$: 67.25% C, 9.57% H, 3.41% N).

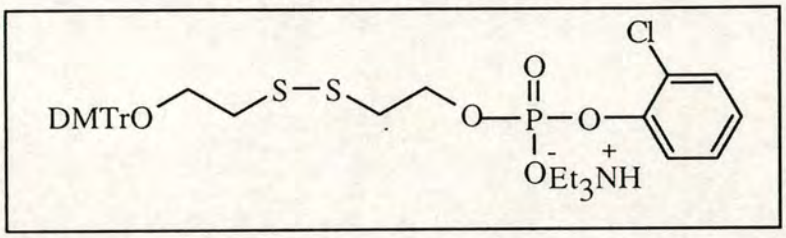
1-(4,4'-Dimethoxytrityloxy)dithiodipropan-1'-O-[(2,5-dichlorophenyl)-1-benzotriazoyl-6-trifluoromethyl]phosphorothioate [45]



Compound **[31]** (1eq., 1.0g, 2.06mmol) was coevaporated twice with anhydrous pyridine and a solution of 2,5-dichlorophenyl-O,O-bis[(6-trifluoromethyl)-1-benzotriazoyl]phosphorothioate[74] (1.5eq., 3.09mmol, 0.2M, 15.47ml) in anhydrous dioxane was added. The mixture was stirred at room temperature for 20 minutes then diluted with dichloromethane (15ml) and washed with brine (2 x 20ml). The organic layer was dried (anhydrous sodium sulphate), then the product was purified by silica-gel flash chromatography, eluting with ethyl acetate in dichloromethane (0-50%) to give a clear oil..

Yield 1.22g (65%). R_f 0.39 (system F). υ_{max}(film)/cm^{-1} 3408 (s, OH), 3067 (w), 2986 (s), 2852 (s), 2685 (s), 2504 (s), 1580 (s), 1474 (s), 1392 (s), 1363 (w), 1251 (m), 1232 (m), 1169 (s), 1126 (s), 1092 (m), 1037 (m), 937 (s), 872 (m), 804 (s), 746 (m). FAB mass spectrum: m/z 910 (M^+). ^{1}H-NMR ($CDCl_3$): δ 1.92-2.02 (m, 4H, 2, 2' CH_2); 2.53-2.75 (m, 4H, 3, 3' CH_2); 3.59-3.66 (m, 2H, 1 CH_2); 3.76 (s, 6H, 2OCH_3); 4.15-4.18 (m, 2H, 1' CH_2); 6.77-8.07 (m, 19H, ArH). ^{13}C-NMR ($CDCl_3$): δ 28.29 (CH_2); 30.11 (CH_2); 34.70 (CH_2S); 36.93 (CH_2S); 54.94 (2OCH_3); 60.99 (CF_3); 65.13 (CH_2O); 65.25 (CH_2O); 112.74 (4ArCH); 120.76 (ArCH); 121.93 (ArCH); 122.76 (ArCH); 123.73 (ArCH); 125.07 ArCH); 126.25 (ArC); 127.48 (ArCH); 128.86 (2ArCH); 129.67 (2ArCH); 130.24 (ArCH); 130.41 (4ArCH); 131.90 (2ArC); 132.36 (2ArC); 139.35 (ArC); 143.87 (ArC); 148.61 (ArC); 148.97 (ArC); 158.18 (2ArC). ^{31}P-NMR ($CDCl_3$): δ 53.04 (s); 55.03 (s).

Triethylammonium[1-(4,4'-dimethoxytrityloxy)dithiodiethan-1'-O-(2-chlorophenylphosphate)] [46]

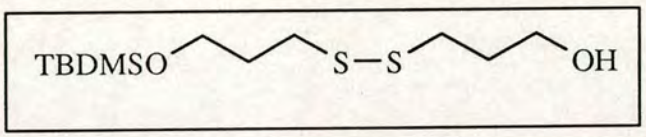


Dithiodiethyl-1-(4,4'-dimethoxytrityl)-1'-ol[126] **[8]** (0.80g, 1.75mmol) was coevaporated twice with anhydrous pyridine then dissolved in anhydrous tetrahydrofuran (30ml). This was added to a solution of 2-chlorophenyldichlorophosphate (1.5eq., 2.63mmol, 0.64g, 0.43ml) and anhydrous triethylamine (3eq., 5.25mmol, 0.53g, 0.73ml) in anhydrous tetrahydrofuran (20ml). After stirring at room temperature for 2 hours, the reaction mixture was quenched with aqueous 1.0M triethylammonium bicarbonate (30ml) and stirred for a further 2 hours at room temperature. Dichloromethane (20ml) was added and the organic layer washed again with aqueous 1.0M triethylammonium bicarbonate (2 x 20ml) and water (20ml). Following drying (anhydrous sodium sulphate) and removal of the solvent under vacuum, the product was purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-5%) + 1% triethylamine to give a clear oil.

Yield 0.94g (71%). R_f 0.28 (system F). υ_{max}(film)/cm^{-1} 3403 (s, OH), 3060 (m), 2932 (s), 2835 (m), 2671 (m), 2483 (s), 2288 (m), 1607 (s), 1585 (m), 1508 (s), 1479 (s), 1445 (m), 1396 (w), 1298 (w), 1248 (s), 1176 (s), 1091 (s), 1061 (s), 1031 (s), 901 (s), 831 (s), 757 (s), 702 (m). FAB mass spectrum: m/z 646.10157 [calc. for $C_{31}H_{32}O_7PS_2Cl$ (M^+): 646.10155]. ^{1}H-NMR ($CDCl_3$): δ 1.12-1.28 (t, 9H, $3CH_3$ Eth, J=7.3Hz); 2.77-2.86 (m, 10H, 2, 2' CH_2, $3CH_2$ Eth); 3.27-3.32 (m, 4H, 1, 1' CH_2); 3.73 (s, 6H, $2OCH_3$); 6.75-7.42 (m, 16H, ArH); 7.59-7.63 (dd, 1H, 5 CH chlorophenyl, $J_{5\text{-}6}$=1.2Hz, $J_{4\text{-}5}$=8.2Hz). ^{13}C-NMR ($CDCl_3$): 9.13 ($3CH_3$); 38.99 (CH_2S); 39.33 (CH_2S); 45.50 ($3CH_2N$); 54.95 ($2OCH_3$); 61.74 (CH_2O); 64.02

(CH_2O); 85.91 (C); 112.81 (4ArCH); 121.26 (ArCH); 123.14 (ArCH); 124.69 (ArC); 126.46 (ArCH); 127.24 (ArCH); 127.52 (2ArCH); 127.88 (2ArCH); 129.52 (ArCH); 129.75 (4ArCH); 135.87 (2ArCH); 144.64 (ArC); 149.09 (ArC); 158.15 (2ArC). ^{31}P-NMR ($CDCl_3$): δ -5.25 (s).

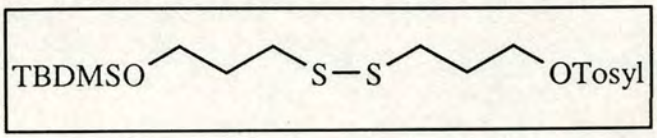
1-(*t*-Butyldimethylsilyloxy)dithiodipropan-1'-ol [47]



Compound **[30]** (1eq., 3.10g, 17.00mmol) was coevaporated twice with anhydrous pyridine then dissolved in anhydrous pyridine (25ml) to which was added *t*-butyldimethylsilyl chloride (1eq., 17.00mmol, 2.56g). The reaction mixture was stirred for 20 hours at room temperature at which point the solvent was removed by evaporation. The residue was dissolved in dichloromethane (25ml) and washed with brine (2 x 15ml). After drying over anhydrous sodium sulphate and concentration by evaporation, the product was purified by silica-gel flash chromatography, eluting with dichloromethane to give a clear liquid.

Yield 3.06g (61%). R_f 0.37 (system E). υ_{max}(film)/cm^{-1} 3364 (s, OH), 2953 (s), 2929 (s), 2866 (s), 1469 (s), 1386 (m), 1360 (w), 1254 (s), 1214 (w), 1154 (w), 1100 (s), 1059 (m), 1007 (w), 947 (s), 835 (s), 776 (s), 717 (w), 663 (m). FAB mass spectrum: m/z 297.13783 [calc. for $C_{12}H_{29}O_2S_2Si$ (M^+ + 2): 297.13782]. ^{1}H-NMR ($CDCl_3$): δ 0.03 (s, 6H, $2CH_3$); 0.87 (s, 9H, $3CH_3$); 1.81-2.00 (m, 4H, 2, 2' CH_2); 2.72-2.81 (m, 4H, 3, 3' CH_2); 3.65-3.77 (m, 4H, 1, 1' CH_2). ^{13}C-NMR ($CDCl_3$): δ -5.52 ($2CH_3Si$); 18.08 (C); 25.72 ($3CH_3$); 31.57 (CH_2); 31.83 (CH_2); 34.82 (CH_2S); 34.92 (CH_2S); 60.63 (CH_2O); 60.99 (CH_2O).

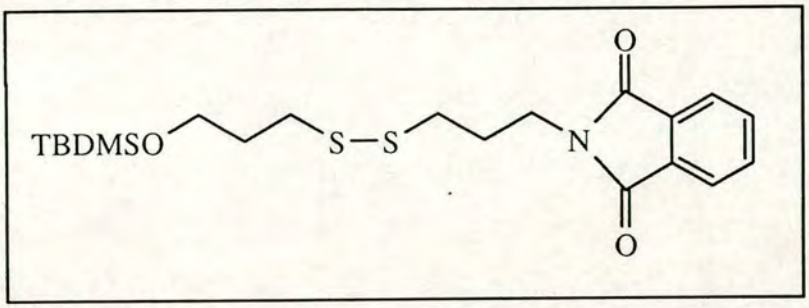
1-(*t*-Butyldimethylsilyloxy)dithiodipropan-1'-O-(*p*-toluenesulphonate) [48]



Compound **[47]** (1eq., 1.65g, 5.58mmol) was coevaporated three times with anhydrous pyridine then dissolved in anhydrous pyridine. The solution was cooled to 0°C and *p*-toluenesulphonyl chloride (1.2eq., 6.70mmol, 1.28g) in anhydrous pyridine (20ml) was added. After stirring for 30 minutes at 0°C, then 3 hours at room temperature, the reaction mixture was evaporated to an oil which was coevaporated twice with toluene. The residue was taken up in dichloromethane (30ml), washed with water (20ml) and brine (3 x 20ml) and then dried (anhydrous sodium sulphate). Removal of the solvent by evaporation was followed by purification of the product by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-5%), to give a clear oil.

Yield 1.85g (74%). R_f 0.74 (system E). υ_{max}(film)/cm^{-1} 2953 (s), 2928 (s), 2855 (s), 1594 (s), 1468 (s), 1377 (s), 1254 (s), 1212 (w), 1187 (m), 1174 (s), 1098 (s), 921 (m), 835 (s), 812 (s), 777 (s), 655 (s). FAB mass spectrum: m/z 452.15453 [calc. for $C_{19}H_{36}O_4S_3Si$ (M^+ + 2): 452.15449]. ^{1}H-NMR ($CDCl_3$): δ 0.02 (s, 6H, $2CH_3$); 0.85 (s, 9H, $3CH_3$); 1.80-2.07 (m, 4H, 2, 2' CH_2); 2.44 (s, 3H, $ArCH_3$); 2.59-2.83 (m, 4H, 3, 3' CH_2); 3.63-3.78 (m, 4H, 1, 1' CH_2); 7.25-7.39 (d, 2H, 2, 6 CH tosyl, $J_{2\text{-}3,6\text{-}5}$=8.4Hz); 7.85-7.89 (d, 2H, 3, 5 CH tosyl, $J_{3\text{-}2,5\text{-}6}$=8.5Hz). ^{13}C-NMR ($CDCl_3$): δ -5.55 ($2CH_3Si$); 18.04 (C); 21.58 ($CH_3$); 25.69 ($3CH_3$); 27.93 ($CH_2$); 31.84 ($CH_2$); 33.54 ($CH_2S$); 34.92 ($CH_2S$); 60.81 ($CH_2O$); 60.93 ($CH_2O$); 126.75 (ArCH); 127.64 (ArCH); 129.71 (ArCH); 130.04 (ArCH); 132.52 (ArC); 146.67 (ArC).

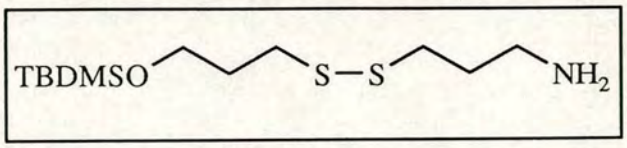
N-Phthaloyl-1'-amino-1-(*t*-butyldimethylsilyloxy)dithiodipropane [49]



To solution of compound **[48]** (1eq., 1.75g, 3.88mmol) in anhydrous N,N-dimethylformamide (15ml) was added potassium phthalimide (1.1eq., 4.27mmol, 0.79g). The reaction mixture was stirred at 55°C for 16 hours, at which point it was diluted with dichloromethane (20ml), washed (water, 2 x 20ml) and dried (anhydrous sodium sulphate). After removal of the solvent by evaporation, the product was purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-5%) to give a waxy, white solid.

Yield 1.48g (90%). R_f 0.73 (system E). υ_{max}(film)/cm^{-1} 3086 (m), 2951 (s), 2928 (s), 2854 (s), 1771 (m, CO), 1714 (s, CO), 1678 (s, CO), 1595 (s), 1466 (m), 1426 (m), 1393 (s), 1360 (s), 1256 (s), 1188 (s), 1008 (m), 947 (w), 836 (s), 777 (s), 717 (s), 690 (w), 660 (s). FAB mass spectrum: m/z 426.15929 [calc. for $C_{20}H_{32}NO_3S_2Si$ (M^+ + 1): 426.15928]. ^{1}H-NMR ($CDCl_3$): δ 0.02 (s, 6H, $2CH_3$); 0.86 (s, 9H, $3CH_3$); 1.83-1.90 (m, 2H, 2 CH_2); 2.04-2.12 (m, 2H, 2' CH_2); 2.65-2.77 (m, 4H, 3, 3' CH_2); 3.63-3.70 (m, 2H, 1' CH_2); 3.74-3.80 (m, 2H, 1 CH_2); 7.67-7.85 (m, 4H, ArH). ^{13}C-NMR ($CDCl_3$): δ -5.46 ($2CH_3Si$); 18.14 (C); 25.77 ($3CH_3$); 28.05 (CH_2); 31.94 (CH_2); 35.12 (CH_2S); 35.76 (CH_2S); 36.68 (CH_2N); 60.98 (CH_2O); 123.11 (2ArCH); 131.90 (2ArC); 133.83 (2ArCH); 168.14 (2CO).

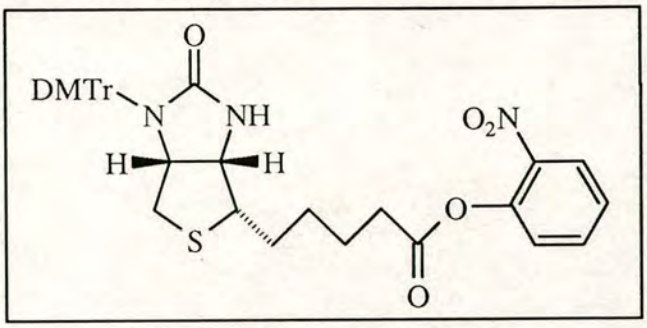
1'-Amino-1-(*t*-butyldimethylsilyloxy)dithiodipropane [50]



To a solution of compound **[49]** (1eq., 3.14g, 7.38mmol) in a methanol/dichloromethane mixture (1:1, 10ml) was added hydrazine hydrate (1.5eq., 11.1mmol, 0.55g, 0.54ml). The mixture was stirred at room temperature for 17 hours, at which point the solvent was removed by evaporation. The residue was dissolved in dichloromethane (20ml), washed with 2M sodium hydroxide solution (20ml) then the aqueous phase extracted with diethyl ether (3 x 20ml). After drying (anhydrous sodium sulphate) and removal of the solvent by evaporation, the residue was purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-10%).

Yield 1.32g (61%). R_f 0.12 (system D). υ_{max}(film)/cm^{-1} 3443 (m, NH_2), 3288 (m, NH_2), 2952 (s), 2927 (s), 2855 (s), 1649 (m), 1469 (s), 1254 (s), 1176 (w), 1099 (s), 1009 (w), 947 (m), 835 (s), 776 (s). FAB mass spectrum: m/z 296.15401 [calc. for $C_{12}H_{30}NO_2S_2Si$ (M^+): 296.15381]. ^{1}H-NMR ($CDCl_3$): δ 0.03 (s, 6H, $2CH_3$); 0.87 (s, 9H, $3CH_3$); 1.79-1.92 (m, 4H, 2, 2' CH_2); 2.68-2.81 (m 6H, 1', 3, 3' CH_2); 3.64-3.70 (t, 2H, 1 CH_2, *J*=6.0Hz). ^{13}C-NMR ($CDCl_3$): δ -5.48 ($2CH_3Si$); 18.14 (C); 25.76 ($3CH_3$); 31.95 (CH_2); 32.78 (CH_2); 35.11 (CH_2S); 36.03 (CH_2S); 40.60 (CH, CH_2N); 61.02 (CH_2O).

[1'-(4,4'-Dimethoxytrityl)]biotin-2-nitrophenylester [51]

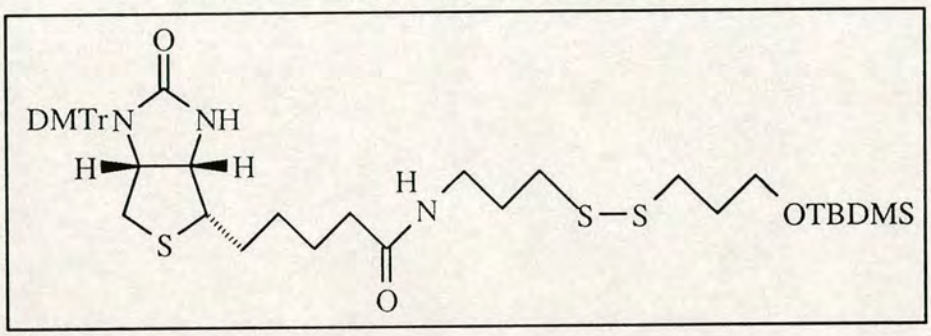


(+)Biotin-2-nitrophenyl ester[157] (1eq., 0.45g, 1.23mmol) was coevaporated twice with anhydrous pyridine then dissolved in anhydrous pyridine (10ml) to which was added 4,4'-dimethoxytrityl chloride (1.2eq., 1.48mmol, 0.50g). After stirring at room temperature for 16 hours, reaction was incomplete and a further 0.18g (0.53mmol) of 4,4'-dimethoxytrityl chloride were added. The reaction mixture stirred for a further 6 hours at which point all the starting material (R_f 0.21, system E) had reacted. The solvent was removed by evaporation, the residue dissolved in dichloromethane (25ml) and washed with water (20ml) and brine (2 x 20ml). After drying (anhydrous sodium sulphate) and concentration under vacuum, the product was purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-5%) primed with 1% triethylamine.

Yield 0.67g (81%). R_f 0.42 (system E). MP 69-71°C. υ_{max}(nujol)/cm^{-1} 3304 (w, NH), 2934 (s), 2837 (s), 1700 (s, CO), 1605 (m), 1531 (s), 1508 (s), 1349 (m), 1298 (w), 1252 (s), 1182 (m), 1112 (w), 1034 (m), 649 (m). FAB mass spectrum: m/z 667.23508 [calc. for $C_{37}H_{37}N_3O_7S$ (M^+): 667.23522]. ^{1}H-NMR ($CDCl_3$): δ 1.41-1.74 (m, 6H, 6, 7, 8 CH_2); 2.18-2.42 (m, 4H, 2, 9 CH_2); 3.07-3.09 (m, 1H, 5 CH); 3.76 (s, 6H, 2OCH_3); 4.27-4.36 (m, 2H, 3, 4 CH); 6.76-7.68 (m, 16H, ArH); 8.04-8.08 (dd, 1H, 5 CH nitrophenyl, $J_{5\text{-}6}$=1.6Hz, $J_{4\text{-}5}$=8.1Hz). ^{13}C-NMR ($CDCl_3$): δ 23.93 (CH_2); 27.83 (CH_2); 28.38 (CH_2); 33.42 (CH_2); 38.88 (CH_2S); 53.94 (CHS); 55.02 (2OCH_3); 59.53 (CHN); 65.22 (CHN); 72.48 (C); 112.61 (4ArCH); 123.53 (ArCH); 125.09 (ArCH); 125.54 (ArCH); 126.36 (ArCH); 126.66

(ArCH); 127.33 (2ArCH); 129.55 (2ArCH); 131.13 (4ArCH); 135.74 (2ArC); 141.61 (ArC); 143.64 (ArC); 149.62 (ArC); 158.17 (2ArC); 161.43 (CO); 170.93 (CO). Analysis: 66.78% C, 5.51% H, 5.81% N (calc. for $C_{37}H_{37}N_3O_7S$: 66.55% C, 5.58% H, 6.29% N).

1'-[1'-(4,4'-Dimethoxytrityl)biotinyl]amino-1-(*t*-butyldimethylsilyloxy)dithiodipropane [52]

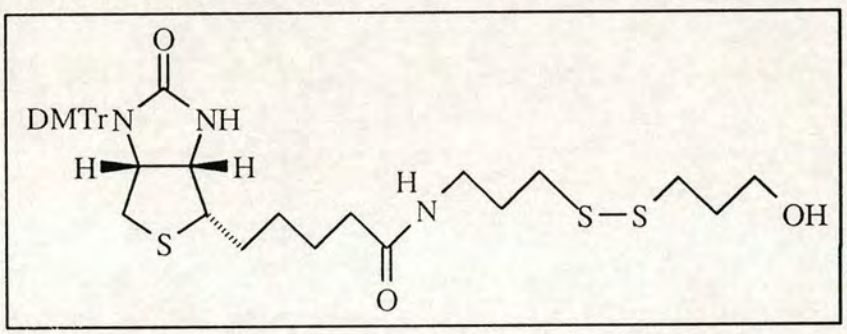


Compound **[51]** (1eq., 0.62g, 0.85mmol) was dissolved in anhydrous N,N-dimethylformamide (8ml) to which was added compound **[50]** (1.1eq., 0.93mmol, 0.25g) and anhydrous triethylamine (1.5eq., 1.28mmol, 0.13g, 0.18ml). After stirring at room temperature for 5 days, the solvent was removed by evaporation and the residue coevaporated twice with toluene and ethanol. The residue was dissolved in dichloromethane (25ml), washed with brine (2 x 20ml) and dried (anhydrous sodium sulphate). After removal of the solvent under vacuum, the product was purifed by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-5%) primed with 1% triethylamine, to give a yellow solid.

Yield 0.42g (60%). R_f 0.29 (system E). MP 79-81°C. υ_{max}(nujol)/cm^{-1} 3306 (m, NH), 3062 (m), 2950 (s), 2930 (s), 2855 (s), 1696 (s, CO), 1606 (m), 1542 (w), 1506 (s), 1419 (s), 1296 (m), 1263 (s), 1218 (w), 1181 (s), 1097 (m), 1034 (s), 945 (w), 833 (m). FAB mass spectrum: m/z 823.35546 [calc. for $C_{43}H_{61}N_3O_5S_3Si$ (M^+): 823.35427]. ^{1}H-NMR ($CDCl_3$): 0.04 (s, 6H, $2CH_3$); 0.87 (s, 9H, $3CH_3$); 1.45-1.55 (m, 4H, biotin 7, 8 CH_2); 1.84-1.91 (m, 6H, 2, 2', biotin 6 CH_2); 2.63-2.74

(m, 8H, 3, 3', biotin 2, 9 CH_2); 3.65-3.70 (m, 4H, 1, 1' CH_2); 3.77 (s, 6H, $2OCH_3$); 6.77-6.81, 7.11-7.29 (m, 13H, ArH). ^{13}C-NMR ($CDCl_3$): δ -5.43 ($2CH_3Si$); 18.14 (C); 25.00 (CH_2); 25.78 ($3CH_3$); 27.78 (CH_2); 28.11 (CH_2); 28.93 (CH_2); 31.95 (CH_2); 35.06 (CH_2N); 35.43 (CH_2); 35.92 (CH_2S); 37.94 (CH_2); 39.14 (CH_2S); 54.24 (CHS); 55.10 ($2OCH_3$); 59.44 (CHN); 61.02 (CH_2O); 65.28 (CHN); 72.56 (C); 112.67 (4ArCH); 126.77 (ArCH); 127.40 (2ArCH); 129.57 (2ArCH); 131.13 (4ArCH); 135.55 (ArC); 135.63 (ArC); 143.58 (ArC); 158.24 (2ArC); 161.48 (CO); 172.82 (CO). Analysis: 63.01% C, 7.40% H, 4.73% N (calc. for $C_{43}H_{61}N_3O_5S_3Si$: 62.66% C, 7.46% H, 5.10% N).

1'-[1'-(4,4'-Dimethoxytrityl)biotinyl]aminodithiodipropan-1-ol [53]

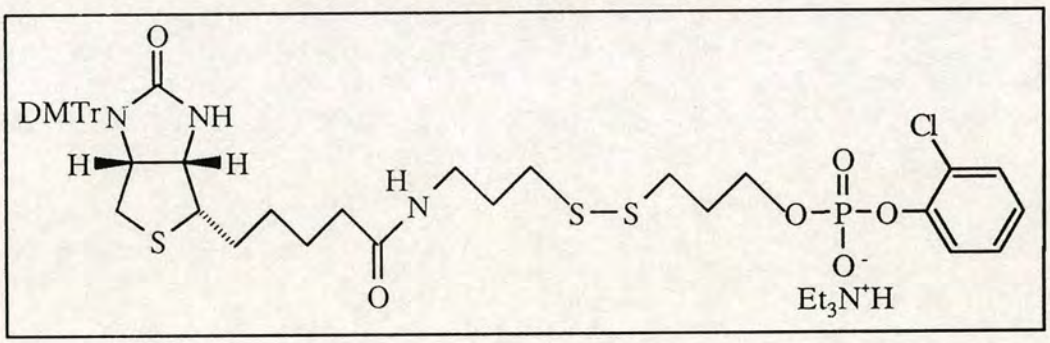


Compound **[52]** (0.35g, 0.42mmol) was dissolved in tetrabutylammonium fluoride in tetrahydrofuran (1.0M, 5ml) and stirred at room temperature for 16 hours at which point all the starting material (R_f 0.75, system D) had reacted. The solvent was removed by evaporation and the residue was coevaporated with toluene and then dissolved in dichloromethane (20ml). The organic layer was washed with water (2 x 10ml), dried (anhydrous sodium sulphate) and the solvent removed by evaporation. The product was purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-5%) primed with 1% triethylamine.

Yield 0.26g (87%). R_f 0.68 (system D). υ_{max}(film)/cm^{-1} 3323 (s, OH), 3085 (m), 2963 (s), 2929 (s), 2835 (m), 1667 (s, CO), 1644 (m), 1607 (m), 1508 (s), 1439 (s), 1295 (m), 1254 (s), 1191 (m), 1064 (m), 1032 (s), 906 (w), 821 (m), 752 (m).

FAB mass spectrum: m/z 709.26521 [calc. for $C_{37}H_{47}N_3O_5S_3$ (M^+): 709.26779]. ^{1}H-NMR ($CDCl_3$): δ 1.42-1.45 (m, 4H, biotin 7, 8 CH_2); 1.76-1.89 (m, 6H, 2, 2', biotin 6 CH_2); 2.23-2.51 (m, 4H, biotin 2, 9 CH_2); 2.61-2.66 (t, 2H, 3 CH_2, *J*=7.1Hz); 2.71-2.76 (t, 2H, 3 CH_2, *J*=7.1Hz); 3.17 (m, 1H, biotin 5 CH); 3.57-3.64 (m, 4H, 1, 1' CH_2); 3.74 (s, 6H, 2OCH_3); 4.22 (m, 2H, biotin 3, 4 CH); 6.74-6.77, 7.06-7.25 (m, 13H, ArH). ^{13}C-NMR ($CDCl_3$): δ 25.00 (CH_2); 27.60 (CH_2); 27.86 (CH_2); 28.70 (CH_2); 31.84 (CH_2); 35.05 (CH_2); 35.21 (CH_2N); 36.09 (CH_2S); 37.73 (CH_2S); 39.10 (CH_2S); 54.41 (CHS); 55.02 (2OCH_3); 59.39 (CHN); 60.21 (CH_2O); 65.10 (CHN); 72.43 (C); 112.55 (4ArCH); 126.68 (ArCH); 127.29 (2ArCH); 129.44 (2ArCH); 131.03 (4ArCH); 135.44 (ArC); 135.51 (ArC); 143.48 (ArC); 158.13 (2ArC); 161.69 (CO); 173.29 (CO).

Triethylammonium{1'-[1'-(4,4'-dimethoxytrityl)biotinyl]aminodithiodipropan-1-O-(2-chlorophenylphosphate)} [54]

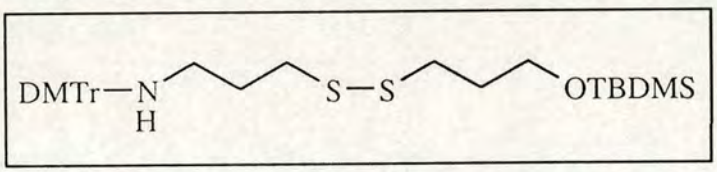


Compound **[53]** (1eq., 0.22g, 0.31mmol) was dissolved in anhydrous tetrahydrofuran (10ml) to which was added 2-chlorophenylphosphate (1.5eq., 0.46mmol, 110mg, 76µl) and anhydrous triethylamine (3eq., 0.93mmol, 94mg, 130µl). The reaction mixture was stirred for 3 hours at room temperature, quenched with aqueous 1.0M triethylammonium bicarbonate (10ml) and stirred for a further 2 hours at room temperature. Dichloromethane (15ml) was added and the organic layer was washed again with aqueous 1.0M triethylammonium bicarbonate (2 x 10ml) and water (10ml) before being dried (anhydrous sodium sulphate). The solvent was removed

under vacuum and the product was purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-5%) + 1% triethylamine to give a waxy, white solid.

Yield 0.15g (48%). R_f 0.32 (system F). υ_{max}(film)/cm^{-1} 3338 (m, OH), 2955 (s), 2930 (s), 2840 (m), 1704 (s), 1508 (s), 1384 (m), 1253 (s), 1182 (m), 1095 (m), 1034 (m). FAB mass spectrum: m/z 899 (M^+). ^{1}H-NMR ($CDCl_3$): δ 0.96-1.02 (t. 9H, 3CH_2 Eth, *J*=7.2Hz); 1.40-1.69 (m, 10H, 2, 2', biotin 6, 7, 8 CH_2); 2.47-2.53 (q, 6H, 3CH_2 Eth, *J*=6.5Hz); 2.67-2.71 (m, 8H, 3, 3', biotin 2, 9 CH_2); 3.31 (m, 1H, biotin 5 CH): 3.52-3.60 (m, 4H, 1, 1' CH_2); 3.78 (s, 6H, 2OCH_3); 4.20-4.26 (m, 2H, biotin 3, 4 CH); 6.78-7.68(m, 17H, ArH). ^{31}P-NMR ($CDCl_3$): δ -5.63 (s).

1'-(4,4'-Dimethoxytritylamino)-1-(*t*-butyldimethylsilyloxy)dithiodipropane [55]

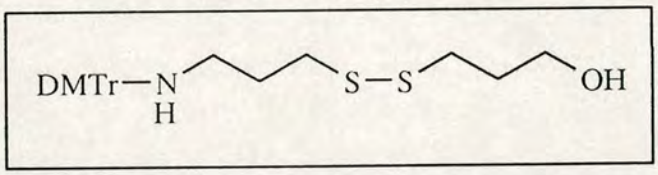


Compound **[50]** (1eq., 0.50g, 1.69mmol) was dissolved in anyhydrous pyridine (10ml) to which was added 4,4'-dimethoxytrityl chloride (1.2eq., 2.03mmol, 0.69g). After stirring at room temperature for 16 hours, the reaction was incomplete and a further 0.17g (0.50mmol) of 4,4'-dimethoxytrityl chloride were added and the reaction mixture stirred for a further 16 hours. All starting material having reacted, the solvent was removed under vacuum and the residue was coevaporated twice with toluene. Without further work-up, the product was purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-5%) primed with 1% triethylamine to give a yellow oil.

Yield 0.53g (52%). R_f 0.86 (system E). υ_{max}(film)/cm^{-1} 3498 (w, NH), 2954 (s), 2928 (s), 2854 (s), 1607 (s), 1581 (w), 1507 (s), 1463 (s), 1442 (m), 1298 (m), 1249 (s), 1212 (w), 1176 (s), 1098 (s), 1035 (s), 946 (m), 910 (w), 832 (s), 776 (s), 702 (m), 664 (w). FAB mass spectrum: m/z 596.26990 [calc. for $C_{33}H_{46}NO_3S_2Si$

(M^+ + 1): 596.26884]. ^{1}H-NMR ($CDCl_3$): δ 0.06 (s, 6H, $2CH_3$); 0.89 (s, 9H, $3CH_3$); 1.83-1.93 (m, 4H, 2, 2' CH_2); 2.19-2.25 (m, 2H. 1' CH_2); 2.72-2.82 (m, 4H, 3, 3' CH_2); 3.67-3.73 (m, 2H, 1 CH_2); 3.78 (s, 6H, $2OCH_3$); 6.78-6.85, 7.16-7.48 (m, 13H, ArH). ^{13}C-NMR ($CDCl_3$): δ -5.43 ($2CH_2Si$); 18.17 (C); 25.80 ($3CH_3$); 30.39 (CH_2); 31.97 (CH_2); 35.19 (CH_2S); 36.77 (CH_2S); 42.23 (CH_2N); 55.02 ($2OCH_3$); 61.04 (CH_2O); 69.70 (C); 112.75 (4ArCH); 125.95 (ArCH); 128.25 (2ArCH); 128.99 (2ArCH); 129.51 (4ArCH); 138.37 (2ArCH); 146.48 (ArC); 157.61 (2ArC).

1'-(4,4'-Dimethoxytritylamino)dithiodipropan-1-ol [56]

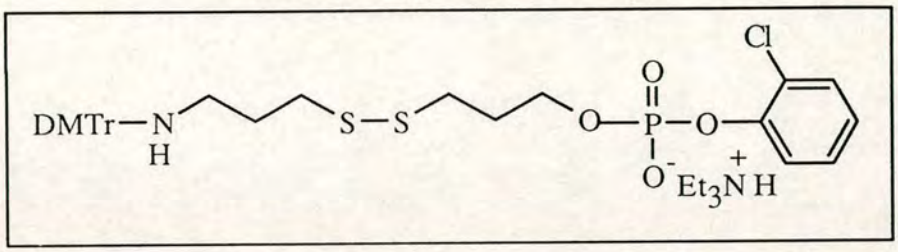


Compound **[55]** (0.21g, 0.35mmol) was dissolved in tetrabutylammonium fluoride in tetrahydrofuran (1.0M, 5ml) and stirred at room temperature for 16 hours. The solvent was removed by evaporation and the residue was coevaporated twice with toluene. Without any further work-up, the product was purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-5%) primed with 1% triethylamine to give a yellow gum.

Yield 73mg (43%). R_f 0.36 (system E). υ_{max}(film)/cm^{-1} 3409 (s, OH), 2949 (s), 2928 (s), 2835 (s), 1606 (s), 1580 (m), 1506 (s), 1443 (m), 1298 (m), 1248 (s), 1177 (s), 1033 (s), 828 (m), 701 (m). FAB mass spectrum: m/z 485.20484 [calc. for $C_{27}H_{35}NO_3S_2$ (M^+ + 2): 485.20584]. ^{1}H-NMR ($CDCl_3$): δ 1.80-1.97 (m, 4H, 2, 2' CH_2); 2.17-2.23 (m, 2H, 1' CH_2); 2.60-2.84 (m, 4H, 3, 3' CH_2); 3.48-3.59 (m, 2H, 1 CH_2); 3.77 (s, 6H, $2OCH_3$); 6.75-6.84, 7.14-7.46 (m, 13H, ArH). ^{13}C-NMR ($CDCl_3$): δ 30.31 (CH_2); 31.72 (CH_2); 35.11 (CH_2S); 36.80 (CH_2S); 42.20 (CH_2N); 55.05 ($2OCH_3$); 60.88 (CH_2O); 69.71 (C); 112.92 (4ArCH); 125.96 (ArCH); 128.24

(2ArCH); 128.99 (2ArCH); 129.51 (4ArCH); 138.36 (2ArCH); 146.46 (ArC); 157.61 (2ArCH).

Triethylammonium[1'-(4,4'-dimethoxytritylamino)dithiodipropan-1-O-(2-chlorophenylphosphate)] [57]



Compound **[56]** (1eq., 92mg, 0.19mmol) was dissolved in anhydrous tetrahydrofuran (8ml) to which was added 2-chlorophenyldichlorophosphate (1.5eq., 0.29mmol, 70mg, 46µl) and anhydrous triethyamine (3eq., 0.57mmol, 58mg, 79µl). The reaction mixture was stirred for 2 hours at room temperature, then quenched with aqueous 1.0M triethylammonium bicarbonate (10ml) and stirred for a further 2 hours at room temperature. Dichloromethane (15ml) was added and the organic layer was again washed with aqueous 1.0M triethylammonium bicarbonate (2 x 10ml) and water (10ml). After drying (anhydrous sodium sulphate), the solvent was removed under vacuum and the product was purified by silica-gel flash chromatography, eluting with methanol in dichloromethane (0-5%) + 1% triethylamine, to give a yellow oil.

Yield 65mg (44%). R_f 0.29 (system F). υ_{max}(film)/cm^{-1} 3402 (s, OH), 2963 (s), 2930 (s), 2836 (m), 2629 (m), 2492 (m), 1648 (m), 1585 (s), 1506 (s), 1444 (s), 1363 (w), 1247 (s), 1177 (m), 1094 (s), 1033 (s), 902 (m), 829 (m). 756 (m). FAB mass spectrum: m/z 673 (M^+). ^{1}H-NMR ($CDCl_3$): δ 1.21-1.27 (t, 9H, $3CH_3$ Eth, *J*=7.3Hz); 1.80 (m, 2H, 2 CH_2); 1.95 (m, 2H, 2' CH_2); 2.14 2.20 (m, 2H, 1' CH_2); 2.66-2.75 (m, 4H, 3, 3' CH_2); 2.91-3.00 (q, 6H, $3CH_2$ Eth, *J*=6.4Hz); 3.75 (s, 6H, $2OCH_3$); 4.04-4.06 (m, 2H, 1 CH_2); 6.74-7.64 (m, 17H, ArH). ^{13}C-NMR ($CDCl_3$): δ 8.72 ($3CH_3$); 30.19 (CH_2); 30.29 (CH_2); 34.96 (CH_2S); 36.73 (CH_2S); 42.21

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Appendix : Anti-HIV Oligonucleotides Synthesised

SRev : d(TCG TCG CTG TCT CCG CTT CTT CCT GCC A)

SRandom : d(CTG GTC TCG TTC GCC TCT CTT CTC CCG A)

SdC28 : d(CCC CCC CCC CCC CCC CCC CCC CCC CCC C)

C35-C6-SRev	C16-SRev	Chol-C6-SRev	HEG-SRev
C35-C6-SRandom	C16-SRandom	Chol-C6-SRandom	
C35-C6-SdC28	C16-SdC28	Chol-C6-SdC28	

Chol-C6-SS-SRev